Title
Combinatorial signaling by twisted gastrulation and decapentaplegic

Permalink
https://escholarship.org/uc/item/1d70k3b1

Journal
Mechanisms of Development, 64(1-2)

ISSN
0925-4773

Authors
Mason, ED
Williams, S
Grotendorst, GR
et al.

Publication Date
1997-06-01

DOI
10.1016/S0925-4773(97)00049-X

License
CC BY 4.0

Peer reviewed
Combinatorial signaling by Twisted Gastrulation and Decapentaplegic

Elizabeth D. Mason, Shawn Williams, Gary R. Grotendorst, J. Lawrence Marsh

Abstract

The Twisted Gastrulation (TSG) protein is one of five secreted proteins required to pattern the dorsal part of the early Drosophila embryo. Unlike the Decapentaplegic (DPP) protein that is required to pattern the entire dorsal half of the embryo, TSG is needed only to specify the fate of the dorsal midline cells. Here we have misexpressed the tsg gene with different promoters to address its mechanism of action and relationship to DPP. When expressed in a ventral stripe of cells, TSG protein can diffuse to the dorsalmost cells and can rescue the dorsal midline cells in tsg mutant embryos. Despite elevated levels that exceed those needed for biological activity, there was no change in dorsal midline or lateral cell fates under any conditions tested. We conclude that TSG does not modulate an activity gradient of DPP. Instead, it functions in a permissive rather than instructive role to elaborate cell fates along the dorsal midline after peak levels of DPP activity have 'primed' cells to respond to TSG. The interaction between TSG and DPP defines a novel type of combinatorial synergism. © 1997 Elsevier Science Ireland Ltd.

Keywords: Secreted protein; Dorsal ventral patterning; Twisted gastrulation; Decapentaplegic; Combinatorial signaling; Permissive induction

1. Introduction

Recent studies in a wide variety of organisms demonstrate that secreted signaling molecules often act combinatorially to specify cell fates during development (for example, Horvitz and Sternberg, 1991; Kimelman et al., 1992; Cornell and Kimelman, 1994a; Munsterberg et al., 1995; Watabe et al., 1995; Theisen et al., 1996). The examples of combinatorial signaling presently known have been classified into antagonistic and synergistic interactions (Cornell and Kimelman, 1994b). Here, we focus on the mechanism of action of Twisted Gastrulation (TSG) and its interaction with Decapentaplegic (DPP) which appears to define a novel class of synergistic interaction.

Patterning of the dorsal/ventral (D/V) axis in Drosophila embryos requires the action of at least five secreted proteins, DPP, Screw (SCW), Tolloid (TLD), Short Gastrulation (SOG), and TSG (Irish and Gelbart, 1987; Shimell et al., 1991; Arora et al., 1994; Francois et al., 1994; Mason et al., 1994). The expression domains of these secreted proteins are broad and overlapping, raising the possibility of direct protein interactions or combinatorial interactions at the level of cell signaling. Since DPP is the only protein that is required for the development of all dorsal structures, it is considered central to dorsal patterning (Arora and Nusslein-Volhard, 1992; Ferguson and Anderson, 1992a) and it has been suggested that a gradient of DPP serves to pattern the dorsal part of the embryo (Ferguson and Anderson, 1992b; Wharton et al., 1993). Consistent with the graded response hypothesis, dpp mutants of increasing severity lead to progressive loss of dorsal structures and shrinking of the dorsal domain with accompanying expansion of ventral ectoderm (Wharton et al., 1993). Mutants of increasing severity in tld and scw also exhibit similar progressive loss of dorsal structures and expansion of ventral ectoderm, suggesting that their normal function may be to enhance DPP activity (Arora and Nusslein-Volhard, 1992; Ferguson and Anderson, 1992a). The phenotype of mutations in sog can be interpreted as if a gradient of DPP were distributed over a broader area resulting in an expansion of dorsal ectoderm at the expense of ventral ectoderm (Ferguson and Anderson, 1992a). SOG is expressed ventrolaterally and restricts DPP
activity to the dorsal half of the embryo, perhaps by binding and preventing DPP from signaling (Francois et al., 1994; Holley et al., 1995; Piccolo et al., 1996). The effects of tsg mutants are unlike tld and scw in that the dorsal midline cells adopt the fates of their more lateral neighbors but the territory giving rise to dorsal structures does not shrink in tsg mutants (Mason et al., 1994). For example, the dorsal midline region, that would normally form the extra-embryonic amnioserosal membrane and other midline-derived structures of the larval head and tail, instead form dorsal ectoderm. Thus, the tsg phenotype gives no hint of affecting a gradient of DPP activity as the other mutants do.

Elevated levels of DPP can suppress or enhance several of the other ventralizing mutations, suggesting that they act upstream of or in parallel with DPP. For example, extra copies of dpp can partially rescue the tld and scw ventralized phenotypes consistent with the model that these proteins act to enhance DPP activity. Elevated levels of dpp in sog mutants enhance the sog mutant phenotype leading to a further expansion of dorsal ectoderm and reduction in ventral denticle band width consistent with the function of SOG protein acting to inhibit DPP signaling activity. Thus, the TLD, SCW, and SOG proteins appear to modulate the levels of DPP activity. In contrast, elevated levels of dpp do not effect even partial rescue of tsg mutants (Ferguson and Anderson, 1992a). The restricted domain of influence of TSG and the failure of extra doses of dpp to override the effects of tsg mutants suggests that TSG interacts differently with DPP than the TLD, SCW, and SOG proteins.

Here, we examine the interaction of TSG and DPP by misexpressing tsg and manipulating levels of tsg and dpp. We demonstrate that the TSG protein is indeed secreted and that the TSG protein can act over a long range (1/2 embryo or ~24 cells). By examining the effects of misexpressing tsg, we conclude that TSG does not modulate an activity gradient of DPP, but instead functions in a permissive rather than instructive role to establish cell fates along the dorsal midline. This interaction between tsg and dpp defines a novel type of combinatorial synergism.

2. Results

To provide a basis for understanding the effects caused by ectopic expression of tsg, we first sought to determine whether TSG is indeed secreted and over what distance the TSG protein can act.

2.1. TSG protein is secreted from insect cells and binds to heparin

The sequence of the tsg gene predicts a hydrophobic N-terminus which could function as a secretory signal sequence (Mason et al., 1994). To confirm this experimentally, we expressed tsg in High Five cells using the baculovirus expression system (Murphy and Piwnica-Worms, 1990). Conditioned medium was collected from control cells infected with wild type virus and from cells infected with the recombinant virus containing the tsg open reading frame at 24 and 48 h after addition of virus. Using a rat polyclonal antibody directed against the TSG protein, no immunoreactive material was detected in the medium from control cells (Fig. 1A, Control). In contrast, the anti-TSG antibody detected a secreted peptide of approximately 28 kDa in the medium of cells infected with the recombinant virus containing the tsg open reading frame 48 h after infection (Fig. 1A, TSG). The 28 kDa peptide detected is similar to the size predicted from the tsg gene sequence (Mason et al., 1994).

Since TSG is distantly related to human connective tissue growth factor (CTGF) (Bradham et al., 1991; Mason et al., 1994) which binds heparin with high affinity (G.R. Groten-dorst, unpublished observations) and heparin-containing glycosaminoglycans may serve as coreceptors for some ligands, we asked whether TSG has affinity for heparin. Conditioned medium containing TSG protein was subjected to affinity chromatography on heparin sepharose. All the immunologically detectable TSG binds to heparin with high affinity and elutes from the column between 0.6 and 0.8 M NaCl at pH 7.4 (Fig. 1B). These results demonstrate that TSG is a secreted protein that binds heparin with high affinity.

2.2. Ventrally expressed tsg rescues tsg mutant embryos

To determine the distance over which TSG can signal and to test its effect on cells other than dorsal midline cells, we expressed the tsg gene on the opposite side of the embryo from its site of action (i.e. on the ventral side). The tsg gene was fused to a 2.9 kb fragment of the twist (twi) promoter which is sufficient to drive wild type twi expression (Jiang et al., 1991). The twi gene is required for mesoderm specification and is expressed in a tight band of 16–18 cells extending along the ventral A/P axis at blastoderm (Thisset et al., 1988). The twi promoter driving the tsg gene was integrated into Drosophila chromosomes using P-mediated germline transformation to produce P{w+mc, twi>tsg} transgenic flies abbreviated here as P{twi>tsg}. Six independent P{twi>tsg} transformant lines were recovered and RNA in situ hybridization to three of these lines showed that the P{twi>tsg} transgene (Fig. 2A) is expressed in the same pattern as native twi RNA (Thisset et al., 1987; Thisset et al., 1988; Leptin and Grunewald, 1990; Jiang et al., 1991). Specifically, tsg RNA from P{twi>tsg} is first seen in a ventral stripe of 16–18 cells at the beginning of cellularization (early stage 5) (Fig. 2A). As gastrulation proceeds, most of the tsg-expressing cells become internalized within the ventral furrow (Fig. 2B,C), leaving an external stripe of 1–2 tsg-expressing cells along the ventral midline (Fig. 2C) until rapid germ band extension nears completion. Throughout gastrulation and beginning germ band extension, cells expressing tsg remain on the surface.
at the anterior and posterior poles (Fig. 2B, arrowheads). For comparison, native tsg expression can be seen in the mid-dorsal region (Fig. 2A,B).

All six transformant lines were tested for the ability to rescue tsg mutants. Three lines showed almost 100% rescue of tsg mutant embryos to adults even though tsg was being expressed on the opposite side of the embryo from its normal site of action. The number of surviving tsg mutant males in the remaining three lines was greater than 70% of expected (Table 1). The twist promoter continues to be expressed in the mesoderm through germ band retraction and then later in the precursor cells of adult muscles (Thistle et al., 1988). In larvae, the twist promoter is expressed in the leg, wing, haltere and genital discs as well as in myoblasts of third instar and early pupa (Bate et al., 1991; Currie and Bate, 1991). Despite this widespread expression of tsg by P[twi>tsg], no developmental defects are seen in the adult fly nor any reduction in viability.

The rescue of tsg mutants by ventrally expressed tsg raises several questions. (1) Is ventrally synthesized TSG protein diffusing to dorsal cells? (2) Are dorsal cells selectively binding TSG? (3) Can elevated levels of TSG expand the domain of cells that adopt dorsal midline fates?

2.3. Ventrally expressed tsg is detected on dorsal midline cells

To monitor the physical location of the TSG protein produced by the P[twi>tsg] transgene, embryos were stained with anti-TSG antibodies and rhodamine-conjugated phal-lolidin which serves to distinguish individual cells of the embryo by binding cytoskeletal actin. Embryos were exam-

2.4. TSG protein from ventrally expressed tsg forms a ventral to dorsal gradient on the surface of somatic cells

When expressed ventrally, TSG protein appears on the surface of all cells (Fig. 3C,D) with the intensity of TSG stain diminishing gradually from ventral to dorsal cells (Fig. 3D). Even after most of the tsg-expressing cells have been internalized during ventral furrow formation, TSG protein is
on dorsolateral cells including cells of the posterior transverse furrow (PTF) at the beginning of rapid germ band extension (stage 8) (Fig. 3G). By the middle of rapid germ band extension, TSG is no longer detectable on the surfaces of dorsal or dorsolateral cells (not shown). Thus, TSG protein, secreted by a band of 18 cells along the ventral A/P axis, diffuses dorsally, and it is detectable as a gradient on the surface of the blastoderm and early germ band extended embryos (early stage 8).

2.5. The extent of the dorsal midline domain is independent of TSG protein concentration

The observations that ventrally expressed tsg rescues tsg mutants and can be physically detected as a ventral to dorsal gradient, indicate that TSG is active even at the point of lowest concentration (i.e. the dorsal-most cells). Thus, all cells located more ventrally and laterally are exposed to even higher levels of TSG than are needed for biological activity. This provides a unique opportunity to determine whether elevated levels of tsg can cause an increase in the extent of the dorsal midline anlage. We asked whether this ventral to dorsal gradient could affect the extent of dorsal midline cell fates in two ways. The first was by monitoring the mitotic behavior of cells at cell cycle 14 and the second was by scoring the number of dorsal midline-derived amnioserosa (ASM) cells in the presence of the P{twi>tsg} transgene in wild type embryos and embryos with genetic backgrounds that either raise or lower the level of DPP activity.

The earliest morphological indication that somatic cells of the embryo have adopted different fates or behaviors is seen after the transition from syncytial to cellular blasto-

Table 1

<table>
<thead>
<tr>
<th>(A) P{twi&gt;tsg} transgene no.</th>
<th>(B) % rescue (no.) y tsg BS/Y; P{twi&gt;tsg}/+</th>
<th>(C) Total flies counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>P{twi&gt;tsg}2-31</td>
<td>72 (27)</td>
<td>173</td>
</tr>
<tr>
<td>P{twi&gt;tsg}2-38</td>
<td>95 (62)</td>
<td>412</td>
</tr>
<tr>
<td>P{twi&gt;tsg}2-109</td>
<td>101 (40)</td>
<td>238</td>
</tr>
<tr>
<td>P{twi&gt;tsg}3-125</td>
<td>75 (26)</td>
<td>231</td>
</tr>
<tr>
<td>P{twi&gt;tsg}3-132</td>
<td>87 (69)</td>
<td>441</td>
</tr>
<tr>
<td>P{twi&gt;tsg}3-186</td>
<td>98 (28)</td>
<td>161</td>
</tr>
</tbody>
</table>

Male flies that contain one copy of the tsg gene under the ventrally expressed twist promoter (Jiang et al., 1991), P{twi>tsg}, were crossed to tsg mutant females: w/Y, P{twi>tsg}/CyO or P{twi>tsg}/TM2 x y tsgBS/FM7c. Six separate P{twi>tsg} insertion lines (A) were tested for tsg mutant rescue (the chromosome linkage of each is designated by { }2-nn or { }3-nn). The percent to which each line can rescue is shown in (B). If rescue were 100%, one would expect 1/2 as many % tsg/ y tsg BS males as +tsg/ w females since only 1/2 would inherit the transgene. Therefore, to calculate the percent rescue, the number of y tsgBS males was divided by the expected number of rescued males (1/2 total number of +tsg/w females). The actual number of y tsgBS/Y, P{twi>tsg}/+ males is shown in parentheses in (B). In (C), the total number of flies scored includes the FM7/w females and FM7/Y males. These balancer flies are not used in calculating the percent expected because of their slightly reduced viability.
derm, at mitotic cell cycle 14. After 13 synchronous divisions, different clusters of cells enter mitotic cell cycle 14 on different schedules. These regions of dividing cells generally correlate with larval fates that are distinct from those of cell clusters that are on different mitotic schedules (Foe, 1989). In wild type embryos, the dorsolateral mitotic domains 1, 5, and 6 are bilaterally symmetrical and can be seen dividing within 5–6 min of each other on either side of the dorsal midline (Fig. 5A). In contrast, none of the dorsal midline cells divide at this early stage of cell cycle 14.

However, in tsg mutants, these dorsal midline cells do divide on the same schedule as their dorsolateral neighbors so that mitotic domains 1, 5, and 6 extend across the midline (Fig. 5B) (Mason et al., 1994). To assess the effect of ventrally expressed tsg on the mitotic behavior of dorsal midline cells, we established a stock of flies in which the only functioning tsg gene is the P[twi>tsg] transgene (w tsgN9/w tsgN9; P[twi>tsg]/P[twi>tsg]) and compared the mitotic domains of these embryos with both tsg mutant and wild type embryos. In transgenic animals, dorsal midline cells do not divide and mitotic domains 1, 5, and 6 are restored to the bilateral symmetry seen in wild type (Fig. 5C). Notably, the number of non-dividing cells separating the bilateral halves of mitotic domains 1 and 5 is similar to the number found in wild type embryos, approximately 3–5 and 9–12 cells, respectively. Thus, the lowest amount of TSG protein from ventrally expressed TSG is sufficient to permit normal mitotic behavior of cells along the dorsal midline but does not result in an expansion of the domains of non-dividing cells. Furthermore, high levels of ventral tsg expression do not affect mitotic domains anywhere else along the ventral to dorsal axis.

As a second measure of the dorsal midline domain, we counted the number of ASM cells (stained with anti-KR antibody for easy identification) in embryos in which the levels of either TSG or DPP had been manipulated. Two extra copies of the dpp gene in a wild type background can increase the number of ASM cells by as much as 2–2.5-fold, while removing one copy of dpp decreases the number of ASM cells (Wharton et al., 1993). Two extra copies of the dpp gene in a tsg mutant has no effect (Ferguson and Anderson, 1992a). If TSG acts to enhance or concentrate the DPP signal, increasing the amount of TSG protein should result in increased numbers of ASM cells. Four copies of the P[twi>tsg] transgene in a normal background does not alter the number of ASM cells (Fig. 6). Adding zero, two, or three copies of P[twi>tsg] to embryos with only one copy of dpp does not change the number of ASM cells, nor does adding 0 or 2 copies to embryos with a partial loss of function allele of dpp. The number of ASM cells appears insensitive to the dose of tsg. On the other hand, changing the levels of DPP leads to a clear change in the number of ASM cells. Regardless of the amount of TSG, embryos with one, two or four doses of dpp have ~50, ~170 and 320 ASM cells, respectively, while embryos with less than one dose of dpp (dpplow, a moderately weak dpp allele) have only ~30 ASM cells. Thus, the number of ASM cells is unaffected by changes in levels of TSG but is highly dependent on the levels of DPP (Fig. 6).

We also tested whether extra copies of P[twi>tsg] could rescue the ASM cells in weak mutant alleles of scw, tld, and sog. These genes are thought to modulate the dorsal activity level of DPP, with SOG acting to antagonize DPP activity, and TLD and SCW acting to enhance DPP activity (Ferguson and Anderson, 1992a). Mutant embryos with two copies of P[twi>tsg] were stained for the presence of ASM cells. In all genotypes tested, homozygous mutants with P[twi>tsg] made the same low number of ASM cells as mutants without the transgene (data not shown). Therefore, extra TSG protein cannot compensate for reduced DPP activity either directly, or in embryos homozygous for weak alleles of other ventralizing mutants whose normal function is to modulate DPP activity. The extent of the dorsal midline domain is independent of TSG concentration.

2.6. Late expression is important for tsg function

We used an 800 bp promoter region of the tld gene (Kirov et al., 1994) to drive tsg expression in order to distinguish early (syncytial/cellular blastoderm) versus late (early germ band extension) requirements of tsg gene function. The tld promoter is expressed early but fades sooner than the twi promoter. Four P[w+tld>tsg] transformant lines, abbreviated P[tld>tsg], were established and tested for their ability to rescue tsg mutants (Table 2). The extent of rescue was variable and low, ranging from 1% to 33% in different lines. Stocks homozygous for tsg where the only source of TSG protein was from the tld promoter were created from two transgene lines and the stocks examined for the number of ASM cells by staining with anti-KR antibodies. Normally, a tsg mutant makes no ASM cells but embryos from these stocks made variable numbers of ASM cells from ~20 cells to wild type in number. Many had an intermediate number of ASM from 50 to 75 cells (Fig. 7E). In contrast, a homozygous tsg mutant stock with two copies of P[twi>tsg] showed a wild type number of ASM cells in all embryos (i.e. ~170) (Fig. 7F).

RNA in situ hybridization to two of these lines showed that under this promoter, tsg is expressed strongly in the dorsal region of the embryo at syncytial blastoderm (Fig. 7A). At cellularization, the pattern refines into four patches along the dorsal A/P axis (Fig. 7B). As the germ band starts to extend, tld expression posterior to the cephalic furrow fades while only the anterior bilateral domain persists with weak expression (Fig. 7C, arrows). At beginning germ band extension, both tld and dpp mRNAs (Fig. 7D) are fading rapidly and are greatly restricted from their initial dorsal on/ventral off patterns seen at early blastoderm (Shiell et al., 1991); whereas the tsg expression remains strong (wild type stains more intensely than the tsg mutant in (Fig.
To detect the persistence of the TSG protein from the *tld* promoter, embryos with two copies of *P{tld>tsg}* were stained with anti-TSG antibodies (data not shown). At cellularization, faint staining of TSG protein was detected on dorsal cells, consistent with the low level of *P{tld>tsg}* expression at this stage, but by the beginning of germ band extension no TSG protein was detected. In contrast,
TSG protein from the twi promoter is detected on dorsal cells as the germ band extends (Fig. 3E,G). Thus, TSG protein from the P[td2>tsg] transgene does not persist as late as TSG from the P[nvi>tsg] transgene. The observation that the twi promoter rescues mutants to 100% viability while the tld promoter rescues poorly suggests that late expression of tsg is important for tsg function.

3. Discussion

To better understand the mechanism of TSG action, we have expressed tsg at different times and in different regions. TSG expressed on the ventral side of the embryo can act at a distance to rescue mutant mitotic domains on the dorsal side (Fig. 5) and to rescue tsg mutant flies to adulthood (Table 1). TSG protein diffuses from the ventral to the dorsal side of the embryo and can be physically detected in a rough ventral to dorsal gradient on the surface of the embryo.

**Fig. 4.** TSG protein is distributed in a ventral to dorsal gradient on the surface of somatic cells. This graph represents the ventral to dorsal distribution of TSG and actin on the cells of the embryo in Fig. 3C; however, other embryos tested, for example the embryo in Fig. 3E, gave similar distributions. Embryos were converted to inverted black and white images. The NIH image 1.60 computer program for the Macintosh was used to generate average gray scale values (which range from 0 to 255) of a given ventral to dorsal region of an embryo. The gray scale values for TSG range from a high of 135 at the ventral region where tsg-expressing cells are located to a low of 24 at the dorsal midline (over a five-fold decrease); whereas the variation in gray scale values for actin remains small (99-135).

**Fig. 5.** Ventrally expressed tsg RNA in tsg mutant embryos restores but does not expand the midline domain of the non-dividing cells. Anterior is to the left; embryos are positioned dorsolaterally. As previously shown (Mason et al., 1994 Fig. 4), mitotic domains 1, 5, and 6 that are normally bilaterally symmetric (A), expand and fuse across the dorsal midline in tsg mutants. (B) Wild type embryo; the line bisects the dorsal midline where cells between mitotic domains 1, 5, and 6 are not dividing. (B) tsg mutant embryo. Midline cells that flank the white line divide as part of domains 1, 5, and 6. (C) Homozygous tsg mutant embryo with two copies of the P[twi>tsg] transgene. Cell division has been restored to normal so that division of midline cells between mitotic domains 1, 5, and 6 is suppressed. Furthermore, the domain of midline cells that are not dividing is similar in size to that in wild type; ectopic TSG from P{twi>tsg} has not expanded this domain.
Fig. 6. Extra TSG protein has no effect on the number of amnioserosa (ASM) cells. Embryos were stained with the anti-KR antibody to mark the ASM cells. ASM cells at germ band extension were scored and averaged for the genotypes listed in the leftmost column. Embryos with four copies of dpp make about twice as many ASM cells as wild type embryos (Wharton et al., 1993; and personal observation). The addition of two copies of the $P^{(twi>tsg)}$ transgene ($tt$) does not result in further expansion of the ASM. When four copies of the $P^{(twi>tsg)}$ transgene are in a wild type background, the number of ASM cells remains the same as in the control wild types. Embryos with one functional copy of dpp ($dpp^{tm4}$, a null allele) make a highly variable number of ASM cells from 0 to 150 (Wharton et al., 1993; and personal observation) which creates a large standard deviation; however, all embryos with one functional copy of dpp with or without $P^{(twi>tsg)}$ give similar ASM averages with similar large standard deviations. Embryos homozygous for a weak dpp allele ($dpp^b$) with two copies or no copies of $P^{(twi>tsg)}$ show no difference in the number of ASM cells. The number of embryos scored for the different genotypes from top to bottom was $n = 10, 30, 20, 93, 198, 163, 21, 29$.

(A) $P^{(tdb>tsg)}$ transgenic lines were isolated (A) and tested for TSG mutant rescue. The percent to which each line can rescue is shown in (B). If rescue were 100%, one would expect 1/2 as many $y^{tm4}$ males as $tsg^w$ females since only 1/2 would inherit the transgene. Therefore, to calculate the percent rescue, the number of $y^{tm4}$ males was divided by the expected number of rescued males (1/2 total number of $tsg^w +$ females). The actual number of $y^{tm4}$ males, $P^{(tdb>tsg)}$ analyzed is shown in parentheses in (B). In (C), the total number of flies scored included the FM7w females and FM7Y males. These balance flies are not used in calculating the percent expected because of their slightly reduced viability.

Table 2

<table>
<thead>
<tr>
<th>genotype</th>
<th># of dpp genes</th>
<th># of twi&gt;tsg genes</th>
<th># of ASM cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>p[23]/p[23]</td>
<td>4</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>n[3-125]p[2-125]</td>
<td>2</td>
<td>0</td>
<td>300</td>
</tr>
</tbody>
</table>

E.D. Mason et al. / Mechanisms of Development 64 (1997) 61–75
Fig. 7. Expression from the \(P\{tld\rightarrow tsg\}\) transgene is greatly reduced at cellular blastoderm and beginning germ band extension. In all panels anterior is to the left. (A) Lateral view of a syncytial blastoderm. Expression of \(tsg\) from \(P\{tld\rightarrow tsg\}\) is intense over the entire dorsal region. (B) Dorsal view of a cellular blastoderm. Native \(tsg\) expression in the mid-dorsal region has refined into five diffuse stripes (arrowheads). Expression of \(tsg\) from \(P\{tld\rightarrow tsg\}\) refines into four dorsal regions, the most intensely staining of which is located anteriorly; the other three regions stain very weakly (arrows). (C) Dorsolateral view of a \(tsg\) mutant embryo at beginning germ band extension (stage 7) showing weaker than wild type stain of native \(tsg\) expression in the mid-dorsal region between the anterior and posterior transverse furrow. The expression of \(tsg\) from \(P\{tld\rightarrow tsg\}\) has been restricted to two regions of weak expression anteriorly and posteriorly (arrows). (D) Dorsal view of a wild type embryo at beginning germ band extension (stage 7) showing weak, restricted DPP expression. (E) A homozygous \(tsg\) mutant embryo with two copies of the \(P\{tld\rightarrow tsg\}\) transgene stained with anti-KR antibody to mark the amnioserosa (ASM) cells (lateral view, dorsal is up). Homozygous \(tsg\) mutant embryos normally make no ASM cells; the addition of two copies of the \(P\{tld\rightarrow tsg\}\) transgene results in a range of ASM cells being made. This embryo has \(-50\) ASM cells. (F) A homozygous \(tsg\) mutant embryo with two copies of \(P\{tld\rightarrow tsg\}\) transgene. The \(P\{tld\rightarrow tsg\}\) transgene results in a wild type number of ASM cells being made (~200).

Despite persistent expression of \(tsg\) by the \(twi\) and \(tld\) promoters later in the embryo and in discs (Bate et al., 1991; Currie and Bate, 1991; Shimell et al., 1991; M. O’Connor, personal communication), no embryonic or adult defects are seen nor is any lethality observed. Similarly, no defects are observed when \(tsg\) is under the control of the constitutively active \(heat\ shock\ 83\) (\(hs83\)) promoter that is expressed during embryonic and larval development (Mason et al., 1984; Xiao and Lis, 1989). Even when flies are grown at \(29°C\) which results in a \(-5\)-fold increase in \(hs83\) promoter activity (Lindquist, 1980), ectopic \(tsg\) has no affect (Mason and Marsh, unpublished observations). In addition, loss of \(tsg\) in mosaic animals in which all adult tissues are mutant for \(tsg\) still allows viable and fertile flies to develop (Zusman and Wieschaus, 1985; Mason and Marsh, unpublished observations). Thus, one can bathe the entire embryo in TSG and ectopically express TSG in discs but only midline cells of the embryo respond. This is an unusual mode of action for a secreted signaling molecule.
3.2. TSG may bind a heparin-containing coreceptor

Ventral expression of \textit{tsg} produces a ventral to dorsal gradient of TSG protein on cell surfaces (Fig. 3). This binding is specific in that only somatic cells bind TSG while presumptive germ cells (pole cells) do not (Fig. 3A,E). The fact that TSG binds heparin raises the possibility that TSG binds a heparin-containing proteoglycan that is present on the surface of all somatic cells in the early embryo. This putative binding could serve a coreceptor function.

Low affinity coreceptor binding plays a role in several ligand receptor interactions. For example, signal transduction by basic fibroblast growth factor, heparin-binding epidermal growth factor, and transforming growth factor-\(\beta\) (TGF-\(\beta\)) are all enhanced by binding of these ligands to heparin-containing proteoglycans that act as coreceptors (Klagsbrun and Baird, 1991; Higashiyama et al., 1993; Lopez-Casillas et al., 1993). Recent studies of adult mutant phenotypes in \textit{division abnormally delayed}, a gene that encodes a proteoglycan (Nakato et al., 1995), show that they are suppressed by extra copies of \textit{dpp} and enhanced by decreased \textit{dpp}. These data suggest that ligand-coreceptor interactions may play an important functional role in fly development.

If TSG is binding a heparin-containing proteoglycan in vivo, TSG must be able to diffuse rapidly in the proteoglycan layer since both its physical movement and action at a distance are rapid. TSG protein is seen over the entire blastoderm less than 30 min after initial detection of \(P[twi>tsg]\) transcription, and 20–30 min later, mitotic domains 1, 5, and 6 divide (Foe, 1989). The failure of pole cells to bind TSG suggests a difference in surface properties, perhaps the absence of a proteoglycan. This may be correlated with the fact that the pole cells are migrating cells that are not part of the embryonic epithelial layer.

3.3. The size of the midline domain is independent of TSG but dependent on DPP

The level of DPP activity establishes the extent of the region that will assume dorsal midline cell fates. Increasing the activity of DPP either by adding copies of \textit{dpp} or by injecting \textit{dpp} mRNA into the embryo expands the number of ASM cells (Ferguson and Anderson, 1992b; Wharton et al., 1993); whereas loss of DPP reduces the number of ASM cells depending on the severity of the \textit{dpp} mutant (Wharton et al., 1993). Thus, the level of DPP sets the extent of midline fates.

What is the role of TSG? If TSG were functioning to modulate DPP activity, elevated levels of TSG should enhance DPP activity and thus rescue some or all of the ASM cells in a weak \textit{dpp} mutant, and/or in weak alleles of other ventralizing genes such as \textit{tdl}, \textit{scv}, and \textit{sog} which themselves function to modulate DPP activity (Ferguson and Anderson, 1992a; Arora et al., 1994; Holley et al., 1995). Furthermore, elevated levels of TSG might expand the number of ASM cells in a wild type and/or cause additional expansion of ASM cells in embryos with four copies of \textit{dpp}. However, extra TSG from \(P[twi>tsg]\) has no effect on the number of ASM cells (Fig. 6) nor on the extent of the midline domain (Fig. 5). Although the concentration of TSG protein from \(P[twi>tsg]\) is lowest on dorsal cells, the amount is still much higher than the amount supplied by the native \textit{tsg} gene since protein from the native gene cannot be detected while TSG secreted from ventral cells is readily detectable on all somatic cells including dorsal cells. Thus, despite ample TSG being present on the surface of the entire embryo, no cell fates are affected. We also examined wild type embryos with four extra copies of the native \textit{tsg} gene to confirm that additional TSG from its native promoter has no effect on the number of ASM cells (Mason and Marsh, unpublished observations). These data, together with the finding that extra copies of the \textit{dpp} gene do not rescue the \textit{tsg} mutant phenotype even partially (Ferguson and Anderson, 1992a), argue strongly that TSG is not functioning to enhance the activity of DPP.

3.4. TSG is a permissive signal whose mechanism of action establishes the order of events

TSG may be the first example of a secreted cytokine specifying a truly permissive signal. In the absence of TSG, DPP signaling specifies dorsolateral cell fates. But DPP is also the signal that sets competency to respond to TSG. Holding the level of DPP constant while changing the location and elevating the level of TSG using \(P[twi>tsg]\) produces no change in the extent of the dorsal midline fates. On the other hand, two extra copies of the \textit{dpp} gene in a wild type embryo causes expansion of ASM cells (Wharton et al., 1993). We propose that high DPP activity sets the competence of the midline cells to respond to TSG which then signals them to adopt midline cell fates. The number of cells competent to respond to TSG can be expanded or contracted by raising or lowering the level of DPP but cannot be changed by altering levels of TSG. On the other hand, TSG signaling is absolutely necessary (but not sufficient) for midline cells to express their preset fate. Thus, TSG is acting as a truly permissive signal.

This permissive model implies an order of events in which DPP must act first or simultaneously with TSG. Several observations suggest that DPP acts first and TSG second. Firstly, the \(P[tdl>tsg]\) and \(P[twi>tsg]\) transgenes initiate transcription simultaneously (Mason and Marsh, unpublished observations). However, the \(P[tdl>tsg]\) transgene stops expression earlier than the \(P[twi>tsg]\) transgene and is much less effective at rescuing \textit{tsg} mutants (Tables 1 and 2). This suggests that late expression of \textit{tsg} is essential for function. Similarly, like \textit{tdl}, expression of \textit{dpp} begins early at syncytial blastoderm but decreases and localizes by beginning germ band extension (Fig. 7D). In contrast, native \textit{tsg} expression continues from syncytial blastoderm, through cellular blastoderm and beginning...
germ band extension (Fig. 7B,C). By comparing these expression patterns, we infer that late expression of dpp may not be necessary for specifying midline cell fates while late expression of tsg is necessary. Unfortunately, manipulation of dpp by heterologous promoters at this early stage is not technically feasible due to the lack of suitably tight promoters and the lethality deriving from misexpression of dpp.

Why would a mechanism evolve in which one signaling molecule serves to prime cell fate and a second secreted signal was required to realize that fate? Proper timing of events or heterochrony is a critical although poorly understood requirement of development (e.g. Rothe et al., 1992; Dolle et al., 1993; Duboule, 1994). Major tissue movements required for dorsal patterning happen in quick order in the Drosophila embryo. The process of gastrulation occurs over 20 min followed by 35 min to complete rapid germ band extension (Campos-Ortega and Hartenstein, 1985). By the middle of rapid germ band extension, genes for dorsal patterning have faded and dorsal patterning has already occurred. It is during the 35 min of rapid germ band extension that ASM cells change shape and shift laterally to allow proper germ band extension. One can speculate that if dorsal midline cells responded immediately to DPP signaling, the cells of the ASM might begin changing shape and shifting before germ band extension had progressed. If DPP primes cells to respond to TSG, it may take several minutes before the concentration of the limiting response factor (e.g. a receptor?) reaches a minimum threshold level. The interaction between TSG and DPP is a unique case where DPP commits a cell to a certain fate and TSG triggers its differentiation. This appears to be a molecular example of Curt Stern’s prepattern and pattern hypothesis (Stern, 1954a; Stern, 1954b).

3.5. Biochemical and developmental similarities between TSG and connective tissue growth factor

TSG is a distant member of a new vertebrate family of secreted growth factors related to human connective tissue growth factor (CTGF) (Bradham et al., 1991; Mason et al., 1994; for review see Bork, 1993). TSG and CTGF share several similarities. CTGF binds heparin in vitro, eluting at the same salt conditions as TSG while in cell culture CTGF binds to a cell-surface heparin-containing proteoglycan (G.R. Grotendorst, unpublished observations). TSG distribution on the surfaces of blastoderm cells is consistent with its binding to a heparin-containing proteoglycan. Other members of the family also bind heparin (Yang and Lau, 1991) suggesting that this may be a common feature for all members of the family. Like TSG, only a subset of cells are competent to respond to CTGF (Frazier et al., 1996).

Vertebrate bone morphogenetic protein-2 (BMP-2) and Drosophila DPP are functional homologs (Padgett et al., 1993). Just as TSG and DPP are both required for patterning the dorsal midline of the Drosophila embryo, both CTGF and BMP-2 RNA transcripts are found during development in regions of mesenchymal condensation to form Meckel’s cartilage and at later stages expression is seen in the osteogenic zones of long bones (Lyons et al., 1990; G.R. Grotendorst and K. Frazier, unpublished observations). The conservation of molecular partners seen with TSG and CTGF provides yet another example of similar signaling molecules being used in different developmental events in vertebrates and insects. It will be interesting to learn if BMP and CTGF act in sequential order like DPP and TSG during mesenchymal condensation and wound repair.

4. Materials and methods

4.1. Expression of tsg in insect cells

The open reading frame of the tsg cDNA was cloned into pBlueBac II transfer plasmid (Invitrogen) and cotransfected with linearized wild-type AcMNPV into Sf9 cells using Lipofectin (Gibco) according to prescribed techniques (Murphy and Piwnica-Worms, 1990). Clones of the recombinant baculovirus were identified using X-gal and were isolated by plaque purification on lawns of Sf9 cells. Each clone was tested for production of TSG by infecting High 5 cells in monolayer and collection of the conditioned media 4 days post-infection. SDS-PAGE, followed by immunoblotting with rabbit anti-TSG antibodies, was used to identify the clones which exhibited the highest levels of TSG synthesis and secretion. Once these clones were identified, spinner cultures of Sf9 cells at a density of 2.5 × 10^6 cells/ml were infected to create a recombinant tsg baculoviral stock. Baculoviral titers, in PFU/ml, were measured by plaque assay as described previously (Murphy and Piwnica-Worms, 1990).

4.2. Heparin sepharose chromatography of tsg protein

Conditioned medium was collected 48 h after infection of High 5 cells with baculovirus expressing TSG protein and centrifuged (5000 × g for 20 min.) to remove cells. The conditioned medium was then treated with 0.5 mM PMSF and ultracentrifuged for 1 h at 30,000 rpm in a Beckman Type 35 rotor at 4°C to remove baculoviral particles. The TSG protein was purified from the conditioned medium by affinity chromatography using porcine heparin conjugated to sepharose beads (Pharmacia, Inc.) The heparin sepharose column was equilibrated in 0.3 M NaCl, 25 mM Tris–Cl (pH 7.2). The conditioned medium was run through the heparin sepharose column at 5 ml/min at room temperature. The column was washed with 10 bed volumes of 0.3 M NaCl, 25 mM Tris–Cl (pH 7.2) to remove non-bound protein, and bound protein was eluted using a gradient from 0.3 M to 1.5 M NaCl in 25 mM Tris–Cl (pH 7.2). Peak fractions containing TSG were determined by immunoblotting.
4.3. Gel electrophoresis and immunoblotting

Cells were grown in shaker cultures to a density of 3.5 x 10^8 cells/ml and infected with the recombinant baculovirus containing the tsg open reading frame under the control of the polyhedron gene promoter at a MOI = 7. Conditioned medium was collected at 24 and 48 h after addition of virus and centrifuged and ultracentrifuged as described above. Electrophoresis was performed using 12% polyacrylamide gels containing SDS (Laemmli, 1970). Immunoblotting was performed by electrophoresing the proteins in the acrylamide gel to a nitrocellulose membrane. The non-specific protein binding sites on nitrocellulose membrane were blocked by incubation of the membrane in a Tris-buffered saline solution (TBS) (100 mM NaCl, 50 mM Tris-HCl, pH 7.4) with 2% non-fat powdered milk (TBS-milk) at 25°C for 1 h. The blocking solution was removed and rabbit anti-TSG antiserum (1:1000 dilution) was added in TBS-milk and 0.01% sodium azide and incubated overnight at 25°C for 1 h. The membranes were then washed three times in TBS-milk, 5 min per wash, and incubated with alkaline phosphatase-conjugated affinity purified goat anti-rabbit IgG (Cappel-Organon Teknika, Durham NC) at 1/~g/ml in TBS-milk at 25°C for 1 h. The membranes were then washed again three times with TBS-milk and twice with TBS. The TSG peptides were then detected using an alkaline phosphatase substrate solution (1 M Tris-HCl, pH 9.0, 0.25 mg/ml nitro blue tetrazolium, 0.5 mg/ml 5-bromo-4-chloro-3-indolyl phosphate) (Sigma) which formed insoluble colored products at the sites of enzymatic activity.

4.4. Construction of tsg vectors and germ line transformation

A twist 2.9 kb promoter region driving the β-galactosidase gene (lacZ) in the pCaSpeR-AUG vector (Pirrotta, 1988; Jiang et al., 1991) was a gift from Mike Levine (Univ. of Calif., Berkeley). The lacZ gene was excised with BamHI leaving 160 bp of the twl 5' UT and with XbaI in the polylinker site 3' to the lacZ gene. A BglII-XbaI tsg transcript region was cut from 1.4 kb tsg pGEM (a CiaI-BamHI genomic fragment) and directionally cloned into the BamHI-XbaI sites of twl-pCaSpeR to produce P[w^{+mC}, twl>tsg].

A 796 bp BamHI/KpnI fragment containing the tld regulatory sequences (Kirov et al., 1994) in pCaSpeR was a gift from M. O'Connor (Univ. of Calif., Irvine). The tld promoter-pCaSpeR construct was cut downstream of the tld promoter with EcoRI and filled in using the Klenow enzyme. A BglII to SspI genomic fragment containing the tsg transcript region was cloned by blunt end ligation after filling in into the filled in EcoRI site of the tld promoter-pCaSpeR construct to produce P[w^{+mC}, tld>tsg]. Diagnostic cuts were performed to ensure correct orientation of the tsg transcript.

Germ line transformation of the transgenes in pCaSpeR (P[w^{+mC}, twl>tsg] and P[w^{+mC}, tld>tsg]) was carried out as described by Spradling (1986). Each construct was co-injected with the helper plasmid P(π)25.7wc (Karess and Rubin, 1984) into embryos collected from a white stock. Multiple independent lines were generated for both constructs.

4.5. In situ hybridizations

Digoxigenin RNA probes were labeled and unincorporated ribonucleotides were removed according to the protocol accompanying the DIG RNA Labeling Mixture (Boehringer Mannheim #1277073). The RNA pellet was air dried, resuspended in 100 µl water and frozen at −20°C. Aliquots of 10 µl were hydrolyzed, precipitated, and resuspended in 100 µl of hybridization solution. For each hybridization, 1–2 µl of hydrolyzed probe was added to a 50 µl total embryo-hybridization solution volume.

Embryos were treated for hybridization as described by Tautz and Pfeifle (1989) with modifications described in Mason et al. (1994). Embryos were mounted in 70% glycerol in 1 x PBT for photography using a Nikon microscope with Nomarski optics, a Nikon camera, and Kodak Tungsten 160 ASA slide film.

4.6. Examination of mitotic domains

Three to 6 h embryos were collected from w{tsg}^{N9} / w{tsg}^{N9}, P{twi>tsg}^{2-109} / P{twi>tsg}^{2-109}, fixed, and stained with anti-tubulin antibody and FITC-conjugated anti-mouse secondary as described (Foe, 1989; Mason et al., 1994). Embryos from tsg mutant stocks and OreR wild type are from Mason et al. (1994). Embryos were viewed as described in Mason et al., (1994). All images were transferred to Adobe Photoshop where orientation and brightness were adjusted.

4.7. Fly stocks and stain of amnioserosa cells

Most mutations used in this work are described in Lindsley and Zimm (1992). Two copies of the P{twi>tsg} transgene were crossed to the following mutants: dpp^{H48}, dpp^{b4}, sog^{YS06}, sog^{XM42}, sog^{YL36}, scw^{IG76}, and tld^{P074}. All stocks contained marked balancers, e.g. FM7cftz > lacZ (FM7c, P[ry^{+72} = ftz/lacZ]); CyO-wglAcZ, (CyO, P[lacZ^{mA}, ry^{+72} = enl]w^{enl}); TM3,hb > lacZ (TM3 P[w^{+mC} = Thb2-lacZ]) and CyO, P[23] wg > lacZ, which is CyO, P[lacZ^{mA}, ry^{+72} = enl]w^{enl} P[dpp^{+10b}, ry^{+72} = dpp-Sal20]2-1 (Mason et al., unpublished) for easy identification of homozygous and heterozygous embryos. Five to 9 h embryos were collected from mutant control stocks and mutant stocks with the P{twi>tsg} transgene. These were fixed, and blocked in PBT-BSA as described for mitotic domain analysis excluding the taxol treatment (Foe, 1989; Mason et al., 1994). Embryos were incubated at 4°C overnight in a 1:100 dilution of a rabbit
polyclonal anti-Kruppel antibody and a 1:1000 dilution of mouse anti-β galactosidase antibody (Promega) in PBT-BSA. The anti-KR antibody was kindly provided by R. Warrior (University of Southern California, Los Angeles). After several rinses in PBT, embryos were incubated at a 1:500 dilution of either peroxidase-conjugated or alkaline phosphatase-conjugated goat anti-rabbit and goat anti-mouse secondaries (Jackson Laboratories) in PBT-BSA overnight at 4°C or 2 h at room temperature. After rinsing again, stain was developed for peroxidase-conjugated secondaries as described in Wharton et al. (1993). Stain for alkaline phosphatase-conjugated antibodies was performed as described by Boehringer Mannheim for alkaline phosphatase-conjugated anti-digoxigenin antibody. Homozygous mutant and heterozygous embryos were sorted by the lacZ marker stain and mounted in 70% glycerol in 1 x PBS for scoring amnioserosa cells using a Nikon microscope, Nomarski optics, and a Sony Trinitron 12-inch monitor. Laterally positioned embryos at germ band extension were viewed on the monitor and ASM cells were scored on one side to the dorsal midline and then doubled to approximate the total number or, when possible, focused through to count the other side. In general, the two methods yielded similar totals.

4.8. Production of TSG antibodies

A PstI restriction fragment beginning near the middle of the tsg coding region and extending through the translation stop codon, was cloned into PstI cut pUC19 (Messing, 1983) and the identity of each construct confirmed by restriction analysis. To analyze expressed proteins, transformed bacteria were grown overnight in LB media, then diluted ten-fold with B-broth to minimize catabolite repression of lacZ expression. After 4 h at 37°C, the cell density was measured, the cells pelleted and lysed by boiling in Laemmli sample buffer (Laemmli, 1970) at a density of 40 OD600 units/ml. Fifteen μl of this solution was analyzed by SDS-PAGE and stained with Coomassie brilliant blue G-250 (Blakesley and Boezi, 1977).

For protein isolation, preparative (3 mm) gels were stained, the desired band cut out and the protein electroeluted from the acrylamide slice into dialysis tubing in Laemmli electrophoresis buffer. The eluate was lyophilized and SDS removed from the pellet by ion-pair extraction (Henderson et al., 1979). The resulting pellet was resuspended in saline and sent to a commercial antibody farm where rats were injected subcutaneously.

4.9. Antibody staining of embryos and analysis of protein distribution

Embryos were collected and fixed in the same manner as they were for RNA in situ hybridization (see above). After rehydrating from methanol to Tris buffered saline-Tween-20 (TBT; 145 mM NaCl, 20 mM Tris–HCl, pH 7.4, 0.1% Tween-20), embryos were blocked in TBT-BSA (1 × TBS, 3% bovine serum albumin, 0.1% Tween-20), for at least 2 h with four changes of TBT-BSA. Embryos were then incubated overnight at 4°C in a 1:100 dilution of preabsorbed anti-TSG antibody in TBT-BSA. Anti-TSG antibodies were preabsorbed for 1 h at room temperature at a 1:10 dilution in TBT with embryos older than 6 h. Embryos were then rinsed for at least 5 h at room temperature or 4°C overnight in TBT-BSA with at least four changes of rinse. Embryos were incubated in TBT with a 1:100 dilution of anti-rat FITC (Jackson Immunological Laboratory) for 2 h at RT or overnight at 4°C. After at least four TBT rinses over a 2 h period, embryos were incubated for 30–45 min in 100 μl of a TRITC-rhodamine conjugated phalloidin in TBT (0.1 mg of powdered TRITC-rhodamine-phalloidin (Sigma P-1951) was stored at 4°C in 1 ml of methanol and 10 μl was vacuum spun dry and resuspended in 100 μl of TBT).

After phalloidin incubation, embryos were rinsed again for 2 h with at least four TBT rinse changes. Embryos were stored and mounted in a glycerol-gallate solution (5% N-propyl gallate, 70% glycerol, 100 mM Tris pH 7.4).

Embryos were viewed with a BioRad Laser Scanning Confocal Microscope Model MRC 600 attached to a Nikon Optiphoto microscope or a BioRad Laser Scanning Confocal Microscope Model 1048 attached to a Nikon inverted scope. Ziess 25 x oil emersion and Nikon 40 x oil emersion lenses were used. Sagittal Z-series were comprised of 10–20 serial sections scanned 0.5–0.7 μm apart for 25 x or 0.2–0.4 μm for 40 x. Surface Z-series only contained 7–12 serial sections scanned at similar μm. Images were transferred to Photoshop where their orientation and overall brightness were sometimes adjusted.

For TSG protein comparisons, embryos from four stocks: OreR or w/w, tsg deficiency (Df(1)RC29/FM7c), w/w flies that contain two copies of P[twi>tsg] transgene and tsg deficiency with two copies of P[twi>tsg], were stained and analyzed. Both the deficiency embryos and wild type embryos appeared identical and showed no TSG protein stain. The deficiency embryos and wild type embryos with two copies of the P[twi>tsg] transgene had strong ventral staining of TSG protein. The presence of TSG protein on dorsal cells was weakly detectable but could often not be seen in merged rhodamine/FITC images. To maximize the visualization of TSG protein on dorsal cells, all the transgenic embryos shown in Fig. 3 are wild type embryos with four doses of the P[twi>tsg] transgene. The RC29/FM7 tsg deficiency stock is unhealthy and weak and it was difficult to create a stock of RC29/FM7 with two copies of the P[twi>tsg] transgene. We tried unsuccessfully to create an RC29/FM7 stock with four copies of P[twi>tsg].

Wild type embryos were always fixed, stained for TSG protein, and analyzed simultaneously with four-dose P[twi>tsg] embryos to ensure that any variations in the experimental procedures did not result in the appearance of an endogenous TSG background. At least ten separate simultaneous staining experiments of wild type and four-
TSG protein was consistently absent from wild type embryos. The distribution of TSG protein and actin was quantified using NIH image 1.60. Confocal images of embryos stained for TSG and actin were converted separately to black and white negative images in the NIH program and dorsal/ventral vertical rectangles were drawn in several places along the anterior/posterior axis of each embryo. The average gray scale values from ventral to dorsal were obtained using 'plot profile' command and graphs were plotted using Microsoft Excel. Graphs were similar among different embryos and within a single embryo (i.e. a ventral to dorsal gradient of TSG protein stain and a constant distribution of actin stain).

Acknowledgements

We wish to thank Mike Levine for his generous gift of the twist promoter clone and Mike O’Connor for the tld promoter clone. Use of the Optical Biology Shared Resource of the Cancer Center Support Grant (CA-62203) at University of California, Irvine is gratefully acknowledged. We gratefully acknowledge the support of NSF grant DC8615701 to J.L.M.; an NIH Research Program Project PO1 HD27173 (P.J. Bryant, director); and NIH grant GM37223 and Fibrogen, Inc. grants to G.R.G. Some of the work presented here was submitted in partial fulfillment of the requirements for the Ph.D. degree by E.D.M. We are indebted to K. Matthews and the National Drosophila Stock Center in Bloomington, IN for stocks; K. Arora and M. O’Connor for stimulating discussions; and H. Mangalam for assistance with the image analysis.

References


