GFP reporters detect the activation of the Drosophila JAK/STAT pathway in vivo

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Abstract

JAK/STAT signaling is essential for a wide range of developmental processes in Drosophila melanogaster. The mechanism by which the JAK/STAT pathway contributes to these processes has been the subject of recent investigation. However, a reporter that reflects activity of the JAK/STAT pathway in all Drosophila tissues has not yet been developed. By placing a fragment of the Stat92E target gene Socs36E, which contains at least two putative Stat92E binding sites, upstream of GFP, we generated three constructs that can be used to monitor JAK/STAT pathway activity in vivo. These constructs differ by the number of Stat92E binding sites and the stability of GFP. The 2XSTAT92E-GFP and 10XSTAT92E-GFP constructs contain 2 and 10 Stat92E binding sites, respectively, driving expression of enhanced GFP, while 10XSTAT92E-DGFP drives expression of destabilized GFP. We show that these reporters are expressed in the embryo in an overlapping pattern with Stat92E protein and in tissues where JAK/STAT signaling is required. In addition, these reporters accurately reflect JAK/STAT pathway activity at larval stages, as their expression pattern overlaps that of the activating ligand unpaired in imaginal discs. Moreover, the STAT92E-GFP reporters are activated by ectopic JAK/STAT signaling. STAT92E-GFP fluorescence is increased in response to ectopic upd in the larval eye disc and mis-expression of the JAK kinase hopscotch in the adult fat body. Lastly, these reporters are specifically activated by Stat92E, as STAT92E-GFP reporter expression is lost cell-autonomously in stat92E homozygous mutant tissue. In sum, we have generated in vivo GFP reporters that accurately reflect JAK/STAT pathway activation in a variety of tissues. These reporters are valuable tools to further investigate and understand the role of JAK/STAT signaling in Drosophila.

Keywords: STAT; JAK; Unpaired; Drosophila; In vivo reporter; Eye; Wing; Antennal and leg imaginal discs; Embryogenesis; Larva; Gene expression; Transgene; Signal transduction

1. Results and discussion

The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is an evolutionarily conserved signaling system that plays essential roles in numerous biological processes in vertebrates and invertebrates, including immunity, hematopoiesis and proliferation (reviewed in Levy and Darnell, 2002). Since Drosophila is highly amenable to genetic manipulations, it has served as an excellent model organism for studying this pathway (reviewed in Hombria and Brown, 2002; Hou et al., 2002). Genetic studies in Drosophila have identified several components of the JAK/STAT pathway, including three cytokine-like Unpaired (Upd) molecules (Upd, Upd2 and Upd3) (Agaisse et al., 2003; Gilbert et al., 2005; Harrison et al., 1998; Hombria et al., 2005); the transmembrane receptor...
Domeless (Dome) (also called Master of Marelle), which is distantly related to the mammalian gp130 cytokine receptor (Brown et al., 2001; Chen et al., 2002); the JAK Hopscotch (Hop) (Binari and Perrimon, 1994), which is most similar to mammalian Jak2; the STAT Stat92E (Hou et al., 1996; Yan et al., 1996), which is homologous to mammalian Stat3 and Stat5; and Socs36E, a member of the SOCS/CIS/JAB family of JAK/STAT negative regulators (Alexander and Hilton, 2004; Callus and Mathey-Prevot, 2002; Karsten et al., 2002). The JAK/STAT signaling cascade is initiated when Upd binds to Dome, causing the receptor to undergo a conformational change. Hop molecules, which are constitutively associated with the Dome cytoplasmic domain, are then able to phosphorylate one another, as well as specific tyrosine sites on the receptor. Cytosolic Stat92E is recruited to these activated receptor sites and is subsequently phosphorylated on a specific tyrosine residue (Y711) by the associated Hop proteins (Yan et al., 1996). Activated Stat92E molecules dimerize and accumulate in the nucleus where they alter the transcription of target genes, such as dome and Socs36E, by binding to specific DNA sequences (consensus TTCNNNGAA) (Bach et al., 2003; Ghiglione et al., 2002; Karsten et al., 2002; Yan et al., 1996).

The Drosophila JAK/STAT pathway regulates many developmental processes, including sex determination, stem cell maintenance, oogenesis, border cell migration, embryonic segmentation, gut development, tracheal development, cell maintenance, oogenesis, border cell migration, embryonic expression patterns. Activation of the JAK/STAT pathway results in increased levels of Stat92E, which is transcriptionally activated by JAK/STAT signaling (Karsten et al., 2002). Socs36E acts as a negative regulator of this pathway, presumably by either blocking Hop activation, or by competing with Stat92E for activated receptor sites (Alexander and Hilton, 2004; Callus and Mathey-Prevot, 2002). The first intron of Socs36E contains a 441 bp fragment with at least two potential Stat92E binding sites (Karsten et al., 2002). We used tandem repeats of this fragment to drive expression of enhanced or destabilized GFP in vivo. We recently employed a similar strategy to generate a luciferase reporter to monitor JAK/STAT pathway activity in vitro (Baeg et al., 2005). Specifically, we generated a 2XSTAT92E-GFP reporter and a 10XSTAT92E-GFP reporter by placing one or five tandem repeats, respectively, of this 441 bp fragment upstream of a minimal heat-shock promoter (hsp) and a cDNA encoding enhanced GFP (Fig. 1A). We also generated a 10XSTAT92E reporter driving expression of destabilized GFP (called 10XSTAT92E-DGF). While enhanced GFP is stable for more than 24 h, the destabilized form is only stable for ~8 h, and is therefore a better temporal marker of transcriptional activity than enhanced GFP (Li et al., 1998). The accuracy of the 2XSTAT92E-GFP and 10XSTAT92E-GFP reporters was confirmed by their embryonic expression patterns. Activation of the Drosophila JAK/STAT pathway results in increased levels and/or stability of the Stat92E protein (Chen et al., 2002; Johansen et al., 2003; Read et al., 2004). In wild type stage 10 embryos, Stat92E protein is detected in stripes (Fig. 1B), which is consistent with Upd and Upd2 expression domains (Gilbert et al., 2005; Harrison et al., 1998; Hombria et al., 2005). Both the 2X- and 10XSTAT92E-GFP reporters are expressed in a similar striped pattern in stage 10 embryos and specifically overlap with Stat92E protein (Fig. 1B’, B” and data not shown). Previous work has demonstrated that JAK/STAT pathway activity is important for the development of polar cells and border cells in the ovary (Beccari et al., 2002; Silver and Montell, 2001), as well as that of posterior spiracles (Brown et al., 2001), hindgut (Johansen et al., 2003), and pharynx (Hombria et al., 2005) in the embryo. We therefore examined the expression of the 10XSTAT92E-GFP reporter in these tissues. In the ovary, upd is expressed specifically in polar cells and in border cells (Beccari et al., 2002; Silver and Montell, 2001). The 10XSTAT92E-GFP reporter is
expressed in polar cells in stage 4–10 egg chambers and in border cells in stage 10 chambers (Figs. 1C and D arrowheads and yellow arrow). In addition, cells neighboring the polar cells also express this reporter, which is expected due to local diffusion of Upd (Figs. 1C and D). Moreover, the posterior spiracles, hindgut and pharynx of a stage 16 Drosophila embryo all specifically express high levels of the 10XSTAT92E-GFP reporter. These results indicate that our STAT92E-GFP reporters are specifically activated by JAK/STAT signaling in the embryo.

We next examined the ability of the 2X- and 10XSTAT92E-GFP reporters to respond to ectopic JAK/STAT signaling during later developmental stages. Wild type third instar larvae carrying a 2XSTAT92E-GFP transgene exhibit minimal GFP fluorescence (Fig. 2A). However, increased GFP fluorescence is observed in 2XSTAT92E-GFP larvae that also carry a hopTum-1 allele, which encodes a hyper-activated Hop protein (Fig. 2B) (Harrison et al., 1995; Luo et al., 1995). Similar results are found in adult stages. Adults expressing the 2XSTAT92E-GFP transgene exhibit GFP fluorescence only in the eye (Fig. 2C). This is not due to auto-fluorescence, as it was not observed in a wild type adult in the absence of the transgene (data not shown). Using the UAS/GAL4 technique, when JAK/STAT signaling is ectopically induced by expression of hop in the fat body of female 2XSTAT92E-GFP transgenic adults, there is a dramatic increase in GFP expression (Fig. 2D) (Brand and Perrimon, 1993). At both larval and adult stages, similar results were obtained with the 10XSTAT92E-GFP reporter (data not shown). Therefore, both 2X- and 10XSTAT92E-GFP reporters are responsive to JAK/STAT pathway signaling in a range of tissues at various stages of development.

1.2. Spatial and temporal characterization of STAT92E-GFP reporters in imaginal discs

We next wanted to examine whether these reporters reflect JAK/STAT pathway activity in imaginal discs. We looked first at the eye disc, since the JAK/STAT pathway has been
best studied in this tissue (Bach et al., 2003; Chao et al., 2004; Reynolds-Kennally and Mlodzik, 2005; Tsai and Sun, 2004; Zeidler et al., 1999). In situ hybridization reveals that upd is expressed at the posterior midline of the eye imaginal disc throughout the first and second instars, but its expression is not detected after early third instar, suggesting that Upd is active in early eye development (Figs. 3A–D, data not shown).

Upd is a secreted molecule that acts cell non-autonomously (Bach et al., 2003; Tsai and Sun, 2004). However, the cells that respond to Upd and activate Stat92E have not yet been identified. We therefore examined the expression of both upd, using an upd-Gal4, UAS-LacZ (upd> LacZ) reporter, and the STAT92E-GFP reporters in the developing eye disc (Tsai and Sun, 2004). Like upd mRNA, the upd> LacZ reporter is expressed at the posterior margin of second and early third instar eye discs (Figs. 3F–H). However, β-galactosidase (β-Gal) protein is also detected in late third instar eye discs (Fig. 3I). Because in situ hybridization shows that upd mRNA is not present in middle and late third instar eye discs (Fig. 3I), the expression of upd> LacZ during this stage is due to the perdurance of β-Gal. Consistent with the expression pattern of upd, 10XSTAT92E-GFP is highly activated in throughout the posterior domain of second instar eye discs (Figs. 3F and G). The 10XSTAT92E-GFP reporter is expressed many cell diameters away from the upd-producing cells at the posterior midline, indicative of Upd’s long-range effects. The expression of the 10XSTAT92E-GFP reporter fades with time, as evidenced by reduced GFP fluorescence in the early and late third instar eye disc (Figs. 3H and I). Since perdurance is a common problem with in vivo reporters, we compared expression of the enhanced and destabilized 10XSTAT92E reporters. In early and late second instar eye discs, both the enhanced and destabilized 10XSTAT92E reporters have similar expression patterns (compare Figs. 3F and G, with Figs. 3Q and R, respectively). This indicates that during early larval stages, cells in the posterior half of the eye disc are continuously responding to Upd. However, the destabilized GFP reporter is not expressed in the third instar eye disc, demonstrating that expression of the enhanced GFP reporter in third instar is due to the perdurance of GFP protein (compare Fig. 3S with Figs. 3H and I).

We next examined the expression of the STAT92E-GFP reporters in other imaginal discs, including wing, antenna, and leg. The pattern of upd mRNA expression in the wing disc has been previously reported and is consistent with the upd> LacZ expression pattern (compare Figs. 3O, P in this study with Figs. 3b, c in Mukherjee et al., 2005). upd> LacZ is expressed in a large domain in the medial dorsal compartment of second instar wing discs (Figs. 3O, P). In third instar, there are five clearly defined domains of upd expression, three in the medial dorsal compartment, one in the anterior margin of the dorsal/ventral boundary and one in the ventral posterior region (Fig. 3P). Activity of the 10XSTAT92E-GFP reporter overlaps with upd expression in both second and third instar wing discs (Fig. 3O). Interestingly, this reporter is not expressed in the wing pouch proper, but rather entirely surrounds it. Similar to what we observe in the eye disc, the 10XSTAT92E-GFP reporter is most strongly expressed in the wing disc during early larval stages (Fig. 3O).
The expression pattern of upd in the antennal disc has not been previously reported. In early second instar, upd mRNA is expressed in the ventral distal antenna, and becomes restricted to two distinct regions (one anterior and one posterior) in the third instar distal antenna (Figs. 3A–D). The upd > LacZ reporter cannot be detected by β-Gal staining in either the antennal or leg disc (Figs. 3J–L). However, the same upd-Gal4 insertion driving expression of UAS-GFP (upd > GFP) is detected in both antennal and leg discs in a pattern that completely overlaps with upd mRNA as detected by in situ hybridization (compare Figs. 3M and N to Figs. 3E and C, respectively). Both the 10XSTAT92E-GFP and 10XSTAT92E-DGFP reporters are expressed in the distal antenna in second instar in a broad pattern that partially overlaps with upd (Figs. 3F, G, Q and R). In third instar, the enhanced GFP reporter becomes concentrated in two domains in the distal ventral antenna (Figs. 3H and I). However, the destabilized GFP reporter is highly expressed throughout second instar (J) and in the dorsal domain of third instar leg discs (K, L). Although upd is expressed in the ventral domain during these time points, we could not detect 10XSTAT92E-GFP reporter activity ventrally (K, L). In second instar wing discs, upd > LacZ is expressed in the dorsal medial domain (O). 10XSTAT92E-GFP overlaps with upd expression and surrounds the wing pouch proper (O). In third instar, upd > LacZ expression has resolved into five domains, three in the dorsal compartment, one at the anterior lateral of the dorsal-ventral boundary and one in the ventral posterior (P). upd > LacZ is not detected by β-gal staining in antennal and leg discs (F–L). However, when the same upd-Gal4 insertion drives expression of GFP (from a UAS-GFP transgene, upd > GFP), GFP is easily observed in leg (M) and antennal (N) discs. (Q–S) Eye–antennal discs expressing two copies of the destabilized 10XSTAT92E-DGFP reporter. In early and late second instar, the 10XSTAT92E-DGFP reporter (labeled STAT-DGFP) is expressed in the posterior half of the eye disc and in the distal antenna in a manner similar to the enhanced 10XSTAT92E-DGFP reporter (compare F and G with Q and R, respectively). However, in third instar eye–antennal discs, the destabilized 10XSTAT92E-DGFP reporter is not expressed (S). Discs-large (Dlg), which marks cell outlines, is in blue (F–S).
reporter is not expressed in the antenna after early third instar (Fig. 3S).

In the leg disc, \textit{upd} mRNA and the \textit{upd}>GFP reporter are expressed in two distinct domains, one ventral anterior and the other dorsal posterior (Figs. 3 E and M). The leg disc exhibits a dynamic pattern of 10X\textit{STAT92E-GFP} expression (Figs. 3J–L). In early second instar, this reporter is expressed throughout the leg disc and becomes restricted to the dorsal domain in second and third instar (Figs. 3J–L). Interestingly, the ventral anterior domain of \textit{upd} expression (Figs. 3E and M) does not have a corresponding region of 10X\textit{STAT92E-GFP} reporter activity in either second and third instar leg discs (Figs. 3K and L). Similar to what is observed in eye and wing discs, the antennal and leg discs have the highest level of JAK/STAT signaling during early larval stages.

1.3. Expression of the \textit{STAT92E-GFP} reporters requires \textit{Stat92E}

To confirm that the \textit{STAT92E-GFP} reporters are responsive to JAK/STAT signaling in imaginal discs, we assessed at the ability of ectopic \textit{upd} to activate the 10X\textit{STAT92E-GFP} reporter. We and others have previously shown that ectopic expression of \textit{upd} using the GMR promoter causes cells anterior to the morphogenetic furrow to undergo additional rounds of mitosis and to upregulate expression of target genes, such as \textit{dome} and \textit{Socs}36E (Bach et al., 2003; Karsten et al., 2002; Tsai and Sun, 2004). Consistent with this finding, in the presence of ectopic \textit{upd} using a GMR-upd transgene, the 10X\textit{STAT92E-GFP} reporter is ectopically activated in the region anterior to the furrow (Fig. 4B). In contrast, the 10X\textit{STAT92E-GFP} reporter is not activated anterior to the furrow in wild type eye discs (Fig. 4A).

To ensure that the 10X\textit{STAT92E-GFP} reporter is activated only by JAK/STAT signaling, we examined its expression in JAK/STAT loss-of-function clones. We used the \textit{stat92E}^{85C9} allele, which is a strong hypomorphic mutation resulting from an R442P substitution (Silver and Montell, 2001). While the \textit{stat92E}^{85C9} mutation is not a protein null, it generates stronger phenotypes than the putative null allele \textit{stat92E}^{6346} (L.A.E., A.A.C. and E.A.B.,
unpublished observations). In \textit{stat92E}^{85C9} mosaic clones, \textit{10XSTAT92E-GFP} expression directly overlaps with wild type tissue within its normal range of expression (Figs. 4C–C'). As expected, GFP is lost from \textit{stat92E}^{85C9} clones in a cell autonomous manner (Figs. 4C–C'). Reporter expression is also lost in \textit{stat92E}^{85C9} clones in a Minute background, in which \textit{stat92E} mutant tissue has a growth advantage over \textit{Minute}+ tissue (Figs. 4D–D') (Morata and Ripoll, 1975). In these discs, \textit{10XSTAT92E-GFP} is expressed only in heterozygous (\textit{Minute}+) tissue, which contains one wild type copy of \textit{stat92E} (Figs. 4D–D'). We obtained similar results for the requirement of \textit{Stat92E} in activation of the destabilized 10XSTAT92E-DFGF reporter (data not shown). The 10XSTAT92E-GFP reporters are therefore activated by JAK/STAT signaling through another positive regulator of this pathway, in this region.

1.4. Discussion

While a number of developmental processes that require JAK/STAT signaling have already been reported, there are likely additional requirements for this pathway that have yet to be identified. We have developed a tool to examine the in vivo activity of the JAK/STAT pathway in a variety of tissues and developmental stages in \textit{Drosophila}. Both the 2X- and 10XSTAT92E-GFP reporters are expressed in the embryo in an overlapping pattern with Stat92E, and, as expected, in a domain slightly broader than \textit{upd} in a variety of imaginal discs. In nearly every disc examined, 10XSTAT92E-GFP reporter activity overlaps with \textit{upd} expression. The one exception is the ventral anterior domain of the leg disc, in which we observe \textit{upd} expression but no corresponding activity of the 10XSTAT92E-GFP reporter. The reason for this discrepancy is unclear as the functional role of the JAK/STAT pathway in leg development is currently not known. However, potential explanations include the lack of \textit{domo} expression, or the lack of another positive regulator of this pathway, in this region. Nevertheless, we demonstrate that when Stat92E is removed, expression of the 10XSTAT92E-GFP reporter is extinguished in an autonomous manner. Conversely, ectopic activation of JAK/STAT signaling leads to the expression of this reporter.

Our reporter is a more sensitive assay of JAK/STAT pathway activation than monitoring \textit{Socs36E} mRNA. \textit{Socs36E} expression patterns have been reported for the embryo, leg, wing and eye imaginal discs, and in the ovary (Callus and Mathey-Prevot, 2002; Karsten et al., 2002; Rawlings et al., 2004). In the embryo and ovary, our GFP reporters and published \textit{Socs36E} mRNA share a very similar expression domain (compare Fig 1 in this study to figures in (Callus and Mathey-Prevot, 2002; Karsten et al., 2002; Rawlings et al., 2004)). However, in imaginal discs, our reporters appear to be more sensitive than \textit{Socs36E} mRNA as detected by \textit{in situ} hybridization (compare Fig. 3 in our study to Fig. 3 in (Karsten et al., 2002)).

Thus, the \textit{STAT92E-GFP} reporters we have developed provide \textit{in vivo} tools to further investigate the JAK/STAT pathway and offer several advantages over other previously published \textit{in vivo} JAK/STAT reporters. First, using our reporters, the \textit{bona fide} activity of Stat92E in a living organism can be monitored by GFP. Second, we developed a destabilized GFP reporter, which is a more accurate temporal marker than enhanced GFP. Third, we document the expression of the 10XSTAT92E-GFP reporter in a wide variety of tissues and developmental stages. In contrast, the expression pattern of other reporters, such as \textit{B}lue-\textit{flu} (Brown et al., 2003), \textit{Stat92E-GFP} (Karsten et al., 2006), and (\textit{GAS})$_T$-\textit{LacZ} (Gilbert et al., 2005), have only been reported in the embryo or in cultured cells. Lastly, our reporters can be used to conduct modifier screens in which mutations can be isolated based on their ability to change the activation of Stat92E rather than on their loss of function phenotype.

2. Experimental procedures

2.1. Drosophila stocks

\textit{hap}$_{\text{Ton-}}$ (Harrison et al., 1995); \textit{yolk-GAL4} (Georgel et al., 2001); \textit{UASH-hop} (Harrison et al., 1995); \textit{stat92E}$_{85C9}$ (Silver and Montell, 2001); \textit{upd-GAL4} (Balder et al., 1995; Tsai and Sun, 2004); \textit{GMR-upd} (Bach et al., 2003); \textit{ey-flp} (Newsome et al., 2000).

2.2. Generation of GFP reporter constructs and transgenic lines

The \textit{STAT92E} reporters were made as described in (Bae et al., 2005), the only difference being that an \textit{Xhol/XbaI} fragment containing a luciferase gene in (Bae et al., 2005) was replaced with an \textit{Xhol/XbaI} fragment containing either enhanced GFP (pEGFP-N1, Clontech) or destabilized GFP (pSE2GF, Clontech). Transgenic animals were generated by standard procedures (Bach et al., 2003). The #6-1 2XSTAT92E-GFP line is a homozygous viable insertion on the 3rd chromosome. The #1 and #2 10XSTAT92E-GFP lines are homozygous viable insertions on the 2nd and 3rd chromosome, respectively. The 10XSTAT92E-DGFP line is a homozygous viable insertion on the 2nd chromosome.

2.3. Mosaic clones

\textit{stat92E} mosaic clones were made by the FLP/FRT technique using \textit{ey-flp}; \textit{P[neoFRT]$_{82B}$P[arm-lacZ.V$^{83B/TM6C}$]}, \textit{Sb}$^b$ \textit{Tb}$^3$ (Xu and Rubin, 1993). Minute clones were generated in the developing eye using \textit{ey-flp}; \textit{P[neoFRT]$_{82B}$P[arm-lacZ.TM6B]}. \textit{ey-flp} is active in the eye disc from early larval stages until late third instar (Newsome et al., 2000).

2.4. \textit{In situ} hybridization, antibody staining, and microscopy

\textit{In situ} hybridization was carried out according to the protocol in (Bach et al., 2003).

We performed antibody staining as previously described (Bach et al., 2003). We used the following primary antibodies: rabbit anti-Stat92E (1:1000) (Chen et al., 2002); 40-1A (mouse anti-Discs large) (1:50) and 4F3 (mouse anti-Discs large) (1:50) (both from Developmental Studies Hybridoma Bank); rabbit anti-\textit{D}-Galactosidase (1:100) (Cappel); 40-1A (mouse anti-\textit{Discs large}) (1:50) (both from Developmental Stud-

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Digital Sight DL-UL camera, a Leica MZ8 microscope with an ophthalmic camera, or a Zeiss Axioskop with a Spot Insight QE camera.

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