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Identification of a *Stat* Gene That Functions in Drosophila Development

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Summary

A Drosophila *Stat* gene (*D-Stat*) with a zygotic segmental expression pattern was identified. This protein becomes phosphorylated on Tyr-704 when coexpressed in Schneider cells with a Drosophila janus kinase (JAK), Hopscotch (HOP). The phosphorylated protein binds specifically to the consensus sequence TTCCCGGAA. Suppressor mutations of *hop*$^{Tum\text{-}l}$, a dominant hyperactive allele of *hop* whose phenotype is hematocyte overproduction and tumor formation, were selected. One of these mutants, *stat*^{HJ}, mapped to the same chromosomal region (92E) as does *D-Stat*, had an incompletely penetrant pair rule phenotype, and exhibited aberrant expression of the pair rule gene *even skipped* (*eve*) at the cellular blastoderm stage. Two D-STAT-binding sites were identified within the *eve* stripe 3 enhancer region. Mutations in either of the STAT-binding sites greatly decreased the stripe 3 expression in transgenic flies. Clearly, the JAK–STAT pathway is connected to Drosophila early development.

Introduction

Experiments during the past several years have identified the components of a pathway, the janus kinase–signal transducer and activator of transcription (JAK–STAT) pathway, through which the occupancy of a cell surface receptor by its polypeptide ligand is reported directly to the nucleus (Darnell et al., 1994; Ihle, 1995; Schindler and Darnell, 1995). The most frequent version of the pathway operates through receptor-bound tyrosine kinases, JAKs, that become activated after receptor–ligand interaction to catalyze phosphorylation on a specific tyrosine of the STAT proteins, which function both as signal transducers and, after dimerization and nuclear translocation, bind to DNA and activate transcription. At least 25 different ligand–receptor complexes signal the nucleus through the STAT molecules. The receptors involved include both those that lack and those that possess intrinsic tyrosine kinase activity (Schindler and Darnell, 1995).

Such a pervasively used signaling mechanism seemed likely to function in invertebrates as well as vertebrates. In fact, the Drosophila protein encoded by the *hopscotch* (*hop*) gene (Perrimon and Mahowald, 1986) has been identified as a member of the JAK family (Binari and Perrimon, 1994). Zygotic *hop*$^+$ expression is required for the proliferation of diploid imaginal cells in larvae (Perrimon and Mahowald, 1986). When both the maternal and zygotic contributions of *hop*$^+$ are removed, segmental defects, frequently deletions of denticle belts from the fifth abdominal segment (Perrimon and Mahowald, 1986), are observed as an imperfect expression of pair rule genes *even skipped* (*eve*) (Binari and Perrimon, 1994) and *runt* (*run*) (Harrison et al., 1995).

A dominant, gain-of-function, temperature-sensitive *hop* mutation, *hop*$^{Tumorous\text{-}lethal}$ (*hop*$^{Tum\text{-}l}$) (Hanratty and Dearolf, 1993), has a single amino acid substitution (Luo et al., 1995), resulting in a hyperphosphorylated kinase (Harrison et al., 1995) that causes leukemia-like defects (Hanratty and Ryerse, 1981). This mutation causes overproliferation of phagocytic plasmatocytes and aggregation of these cells into large melanizing tumors in hematopoietic and gut tissue.

We now report a Drosophila STAT protein with high amino acid identity to the mammalian STAT proteins. The gene for this protein, *D-Stat*, maps at 92E on chromosome 3 and is expressed in early embryos in a weak seven-stripe pattern and at both terminal regions. A single tyrosine residue, 704, becomes phosphorylated, and the activated D-STAT can bind DNA when *D-Stat* is expressed in cells along with *hop* or if the *D-Stat*-transfected cells are treated with vanadate to inhibit phosphotyrosine phosphatases. To connect D-STAT to development, we recovered a mutation in the *stat* locus from a genetic screen for dominant suppressors of *hop*$^{Tum\text{-}l}$ lethality. This mutation, termed *stat*^{HiJak} (*stat*^{HJ}), causes reduced viability when homozygous and exhibits a partially penetrant pair rule phenotype, one characteristic of which is aberrant EVE expression at the cellular blastoderm stage.

The early expression pattern of pair rule genes such as *eve* and *run* represent the first evidence of a metameric body plan during Drosophila development (Ingham et al., 1985; MacDonald et al., 1986; Frasch et al., 1987; Gergen and Butler, 1988). Separate enhancers (Stanojevic et al., 1991; Small et al., 1992, 1996) in the *eve* promoter apparently integrate positional information provided by the earlier expressed maternal and gap genes (Goto et al., 1989; Harding et al., 1989). Bicoid (BCD) and Hunchback (HB) serve as activators of stripe 2 expression, and the anterior and posterior borders of the seven stripes are formed by repression mediated by the gap proteins Giant (GT) and Krüppel (KR) (Stanojevic et al., 1989; Small et al., 1991).

Repression to form the borders of stripe 3 is also thought to be mediated by repression carried out by the gap proteins HB and Knirps (KNI) (Small et al., 1996),

which bind to multiple sites within the 500 bp stripe 3 enhancer. However, candidate transcription factors that might activate stripe 3 have not yet been identified. We now report that *D-Stat* functions in stripe 3 expression. Thus, the D-STAT protein appears to play an important role in the segmental pattern formation in the early embryo by activating specific stripes of pair rule gene expression, and from these and the earlier experiments on HOP (Binari and Perrimon, 1994) it appears that the JAK–STAT pathway is active in Drosophila development.

Results

Recovery of Drosophila cDNA Encoding a D-STAT-like Protein

To search for a possible *Stat* gene in Drosophila, an embryonic (0–24 hr) cDNA library was screened by polymerase chain reaction (PCR) amplification of possible STAT-coding sequences. Degenerate oligonucleotides representing the most highly conserved sequences (the src homology domain 2 [SH2] region and the putative SH3 region) of the mammalian STAT proteins were prepared. By PCR amplification, a DNA fragment that matched the mammalian consensus *Stat* sequence in 18 out of 34 positions was cloned and sequenced (residues 619–653; Figure 1A). Using this fragment, two full-length cDNA sequences were recovered that differed in the region of residue 700 (Figure 1A, shaded residues). Seven additional amino acids, possibly from an included extra exon, were encoded in the longer form (inserted at residue 698), near the carboxyl end of the SH2 domain. Mammalian *Stat* genes have many exons (>20), some encoding as few as 14 amino acids, and differential splicing among these exons has been reported (Yan et al., 1995). The Drosophila protein was about 33% identical to Stat5 and Stat6, with 25%–29% identity evident to other STAT family members. The highest regions of identity were in the SH2 domain, where the greatest similarity also occurs in mammalian proteins (Figure 1B). The STAT SH2 domain functions in binding the protein to phosphotyrosine on an activated receptor (Greenlund et al., 1994) and in forming homodimers and heterodimers within the STAT family (Shuai et al., 1994; Qureshi et al., 1995). There was also a tyrosine located just downstream (residue 704 or 711) from the putative SH2 domain in the same position as the phosphorylated tyrosine in mammalian STAT proteins. Other regions of amino acid similarity between the D-STAT and mammalian proteins were found throughout the length of the coding sequences, as is the case for the mammalian proteins. These results indicated that we had recovered the sequence of a Drosophila *Stat* molecule, which we refer to as *D-Stat*.

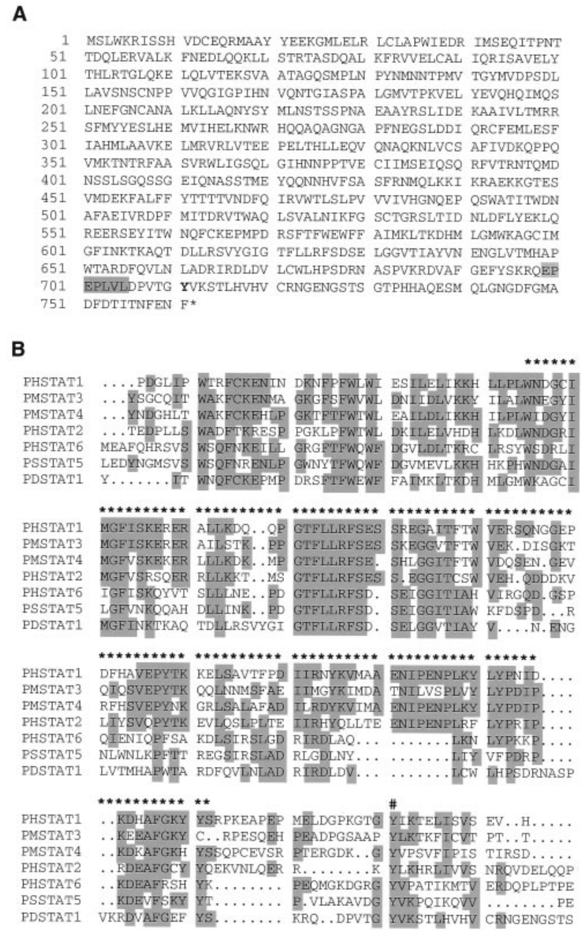


Figure 1. The Amino Acid Sequence of D-STAT and the Alignment of Its Sequence with Known STAT Family Members

(A) The amino acid sequence deduced from the sequence of *D-Stat* cDNA. The shaded sequence represents a longer form of D-STAT protein that contains an additional seven amino acid residues, possibly as a result of differential splicing. The tyrosine residue that is phosphorylated is indicated at 704 in the short form, but at 711 in the longer form D-STAT protein. Only the short form of D-STAT has been used in experiments described here.

(B) The sequence alignment of the most conserved region of the STAT proteins is the SH2 domain. The Y residue that is phosphorylated is marked with a number sign.

Chromosomal Mapping of *D-Stat*

To map *D-Stat* to a specific chromosomal locus, we hybridized a digoxygenin-labeled RNA probe complementary to *D-Stat* to a chromosomal squash prepared from the salivary glands of third instar larvae. One band was observed in chromosome 3 (Figure 2A). This was located precisely by hybridization along with a marker, *Hairless* (92E14–92E15; Bang and Posakony, 1992) close to the region that *D-Stat* mapped by chromosomal morphology. When both probes were used, two bands were seen assigning *D-Stat* to the 92E2 region of chromosome 3 (Figure 2B).

D-STAT mRNA Expression

Total RNA isolated from different embryonic stages, larvae, pupae, and adult male and female flies was examined by Northern blot analysis, revealing a single ~4 kb mRNA in very early embryos and in all stages of embryogenesis and adult flies, with some decrease in concentration in the late embryonic stages (data not shown). The embryonic cell pattern of *D-STAT* expression was then determined by in situ mRNA hybridization on whole embryos. Unfertilized and just fertilized eggs

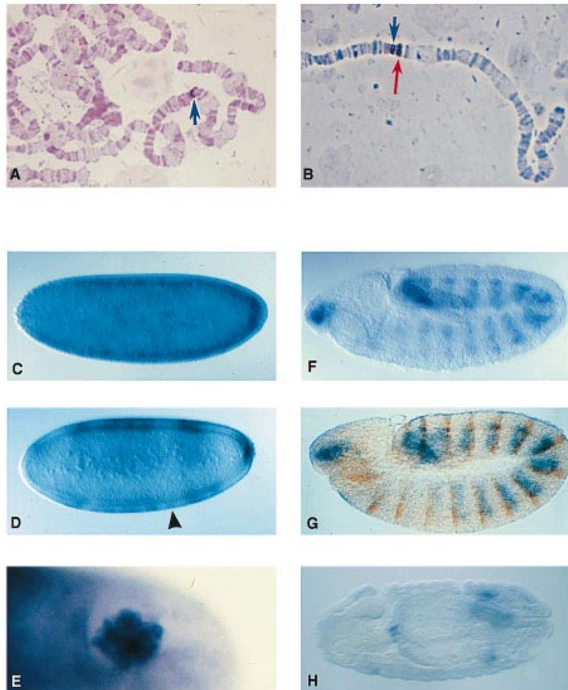


Figure 2. The Localization of *D-Stat* at Salivary Gland Chromosome Band 92E2 and Expression of *D-STAT* during Drosophila Embryonic Development

(A) The *D-Stat* signal is indicated by blue arrow.

(B) The red arrow indicates the localization of the marker gene Hairless.

(C)–(G) show in situ detection of *D-STAT* mRNA in Drosophila embryos.

(C) 1 hr after fertilization.

(D) Cellular blastoderm stage. Weak seven-stripe pattern is seen, with weakest staining at stripe 5, as indicated by arrowhead.

(E) After the beginning of gastrulation, pole cells are strongly stained with the *D-STAT* antisense riboprobe.

(F) *D-STAT* expression in 14 stripes in the somatic mesoderm during gastrulation (about stage 8), with strong staining in gonadal somatic cells, but not pole cells.

(G) Double staining with monoclonal antibody against Engrailed (brown staining); blue staining is *D-STAT* mRNA, with stripes wider than those of Engrailed.

(H) At late embryonic stage, expression of *D-STAT* is concentrated in the regions of foregut, hindgut, and gonadal tissues. All the embryos shown here were oriented with anterior at the left and dorsal up.

(1 hr after laying) stained darkly in a uniform manner, indicating maternally deposited mRNA (Figure 2C). At the blastoderm stage (1–3 hr), zygotic expression started as a broad central domain, clearly showing seven partially separated stripes reminiscent of the expression pattern of many pair rule genes. Stripe 5 stained somewhat less strongly than the other stripes (Figure 2D). In addition to the stripes, clusters of cells in both anterior and posterior terminal segments were stained (about 3.5 hr) (Figure 2E). At germband extension, a segmental pattern of 14 stripes, restricted to mesodermal tissue, was observed (Figure 2F). The anterior boundary of these stripes corresponded to the anterior Engrailed-expressing cells, therefore overlapping the segment boundary. After germband retraction (stage 15), *D-STAT* was expressed mainly in hindgut and foregut, as well as in gonadal precursor cells (Figure 2H).

D-STAT Is Phosphorylated on a Single Tyrosine Residue at 704

We next determined whether the protein encoded by the *D-Stat* cDNA could be phosphorylated in tyrosine and then bind DNA specifically. Ligand-induced activation of STAT proteins in mammalian cells has been tested simply in either of two ways: by specific antibody precipitation of STAT protein in extracts of cytokine-treated cells, followed by detection of phosphotyrosine with anti-phosphotyrosine antibody in the correctly sized protein (Sadowski et al., 1993) or, second, by binding of activated STAT molecules to specific DNA sequences assayed by electrophoretic mobility shift assay (EMSA). Since we failed to obtain rabbit anti-D-STAT antibodies that would precipitate D-STAT, we added a peptide tag, the FLAG epitope, to the vector encoding the recombinant D-STAT protein and used precipitated proteins with monoclonal anti-FLAG antibody (M2; IBI Kodak). A second problem in attempting to study phosphorylation of D-STAT was lack of knowledge about any receptor-mediated tyrosine phosphorylation of this protein. To overcome this difficulty, we have used either of two approaches. First, coexpression of mammalian STAT protein with any mammalian JAK was known to lead to at least some tyrosine phosphorylation of the STAT molecule (Quelle et al., 1995). In addition, treatment of mammalian cells with the tyrosine phosphatase inhibitor sodium orthovanadate leads to "spontaneous" nonligand-mediated tyrosine phosphorylation of STATs in mammalian cells (Volberg et al., 1992; Larner et al., 1993; Zhong, 1995). Thus, we transfected the epitope-tagged *D-Stat* into Schneider cells and either treated them with vanadate or simultaneously transfected a *hop* expression vector to provide excess JAK activity. No detectable tyrosine phosphorylation of D-STAT expressed alone was observed in Schneider cells (Figure 3A, lane 2), but coexpression of the HOP protein (Figure 3A, lanes 4 and 5) or vanadate treatment (Figure 3A, lane 3) led to easily detectable tyrosine phosphorylation on the coexpressed STAT protein. The strength of the signal was increased with an increase of cotransfected *hop* DNA (Figure 3, lanes 4 and 5).

In mammalian cells, STAT proteins are known to be phosphorylated on single Y residues just downstream of their SH2 groups (Schindler and Darnell, 1995). While wild-type D-STAT protein was tyrosine phosphorylated in vanadate-treated cells (Figure 3B, lane 9), protein carrying a mutation of Tyr-704 (the Y residue in the position homologous to mammalian phosphotyrosines) to phenylalanine was not tyrosine phosphorylated (Figure 3B, lanes 8 and 9). Thus, the change of a single tyrosine residue removed all detectable tyrosine phosphorylation of D-STAT.

Binding Site Selection for an Activated D-STAT

Since D-STAT could be tyrosine phosphorylated, we sought to investigate the DNA binding properties of the activated protein. Labeled DNA probes (such as M67; Wagner et al., 1990) that bind a large number of mammalian STATs (Horvath et al., 1995; Seidel et al., 1995) did not bind to extracts containing the phosphorylated

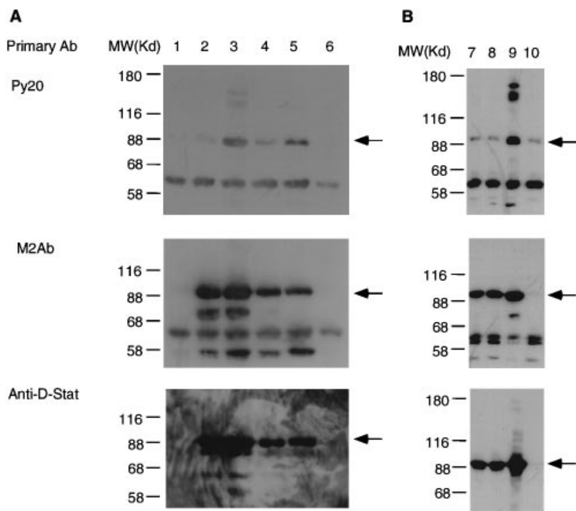


Figure 3. Tyrosine Phosphorylation of D-STAT in Cultured S2 Cells

(A) Tyrosine phosphorylation of D-STAT. Each dish of S2 cells was transfected with the indicated DNAs as follows: 20 μg of pPAC5C-PL vector DNA (lane 1); 20 μg of FLAG-tagged *D-Stat* DNA construct (lanes 2 and 3); 10 μg of FLAG-tagged *D-Stat* DNA plus 5 μg (lane 4) or 10 μg (lane 5) of *hop* DNA; or 10 μg of *hop* DNA alone, supplemented with 10 μg of vector DNA (lane 6). In lane 3, transfected cells were stimulated with vanadate–H_2O_2 mixture for 15 min. The primary antibodies used for Western blot analysis are indicated on the left side of the corresponding panels (Py20, phosphotyrosine antibody; M2Ab, monoclonal antibody against FLAG epitope; anti-D-STAT, antibody against 119 terminal amino acids of D-STAT. The arrow indicates D-STAT protein, which was verified in (B) with anti-FLAG antibody M2 (100 ng/ml; IBI Kodak) and antibody against D-STAT. Both the faint band right above D-STAT protein and the strongly stained band below the D-STAT protein in Py20 blot are nonspecific reactive proteins and present in all lanes.

(B) Mutation of Tyr-704 to Phe-704 abolished the tyrosine phosphorylation of D-STAT. A DNA construct (10 μg) with the tyrosine to phenyalanine mutation at 704 was transfected into S2 cells for 60 hr and stimulated with vanadate–H_2O_2 mixture for 15 min (lane 7), or cells were cotransfected with the mutant DNA and 10 μg of *hop* DNA (lane 8). The wild-type *D-Stat* was transfected and cells were vanadate treated as a positive control in lane 9. Lane 10 was transfected with wild-type protein, but was not vanadate treated.

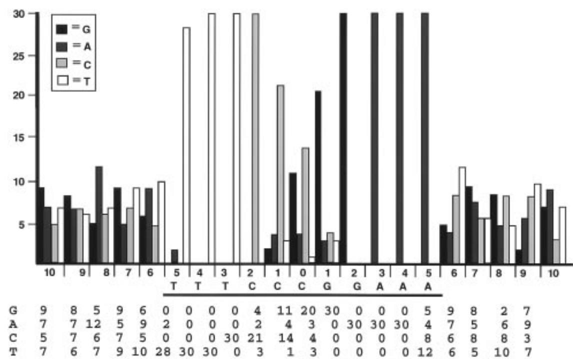


Figure 4. Optimum Binding Site Selection for Activated D-STAT and Determination of the Minimum Sequence Requirement for the Binding of the Activated D-STAT

Sequences of 30 samples derived from cloning DNA fragments after six rounds of binding site selection (Pollock and Treisman, 1990). The most frequently selected sequences in the core region are displayed and underlined. The frequency of each residue in each position is given below the corresponding bars.

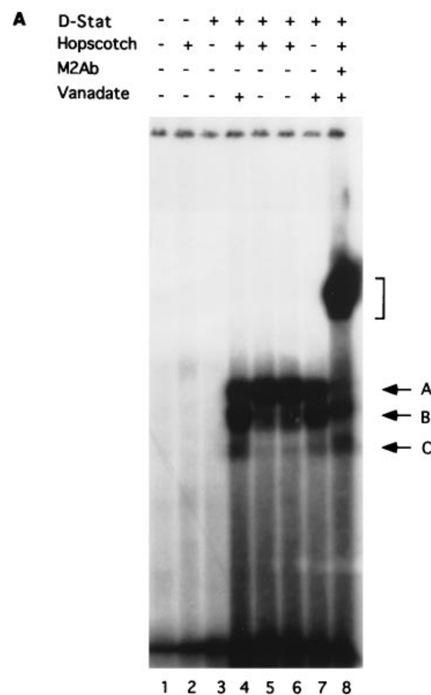
D-STAT. To select a specific D-STAT DNA-binding sequence, we employed a variation of the Pollock and Treisman (1990) technique, using antibody precipitation of the FLAG-tagged D-STAT and PCR amplification of associated oligonucleotides (see Experimental Procedures). We sequenced 30 individual clones prepared from *D-Stat*-associated oligonucleotides. A consensus sequence (Figure 4, TTTCCCGGAAA) was evident that resembled the optimum site selected by Stat1 and Stat3. Mutation of either position 4 (first C) or 8 (last G) of the consensus sequence virtually abolished the binding to the active D-STAT (data not shown). Mutations at positions other than TTC (2, 3, and 4) or GAA (8, 9, and 10) did not substantially affect the D-STAT gel mobility shift. Therefore, the dyad symmetric sequence TTCnnnGAA affords maximal D-STAT binding.

Phosphorylation of Tyr-704 Is Required for DNA Binding

Since a specific DNA oligonucleotide bound to activated D-STAT, we used the EMSA assay to test whether activation by either HOP or vanadate would induce DNA binding activity. Without D-STAT transfection, or in the absence of activation by HOP or vanadate, extracts of Schneider cells exhibited little or no binding of the D-STAT consensus sequence (Figure 5A, lanes 2 and 3). When cells were transfected with FLAG-tagged *D-Stat* and activated by vanadate, three gel shift bands (labeled A, B, and C in order of increasing mobility in Figure 5) were consistently observed (Figure 5A, lanes 4–6). When treated with the anti-FLAG antibody, two of these complexes, A and B, were greatly decreased with the appearance of a supershifted band. Extracts of cells that had been transfected with *D-Stat* and *Hop* and bound to the consensus probe produced one band, labeled A (lanes 5 and 6 in Figure 5A) as the most predominant gel shift complex. Thus, combining the results from both vanadate and HOP activation, we concluded that the activated D-STAT formed the A complex and contributed to the B complex and that the C complex, which was weakly present in the absence of transfection of *D-Stat* (compare lane 1 with lanes 2 and 3 in Figure 5B), could represent a STAT-like protein in Schneider cells. Evidence that other Drosophila STAT proteins may exist also came from cells transfected with the D-STAT Tyr-704→Phe mutant and then treated with vanadate (Figure 5B, lane 8). Proteins in cell extracts without transfected *D-Stat* formed mainly the faster migrating C complex; extracts of cells transfected with the Tyr-704→Phe mutant also did not form complex A, which identified the Tyr-704 residue as necessary for DNA binding activity (Figure 5B, lane 8). Thus, in analogy with mammalian cells, in which two Stats are simultaneously activated (Zhong et al., 1994b; Qureshi et al., 1995), it seems possible that band A is a homodimer of D-STAT, C may be a homodimer of another Drosophila STAT protein, and at least some of the B band is a heterodimer of D-STAT and the additional STAT-like vanadate-inducible protein.

stat^{HJ}, a Mutant of *D-Stat*, Affects Pair Rule Function

To determine whether the D-STAT proteins could be connected to early development, we took two approaches. In the first, we obtained a mutation, termed



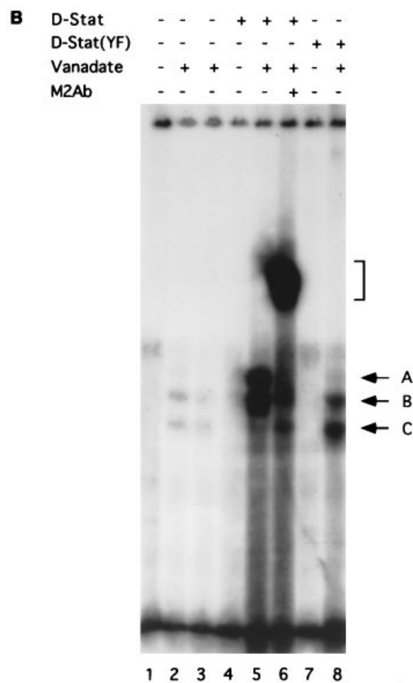


Figure 5. EMSA Analysis of Artificially Activated D-STAT Proteins

(A) HOP activates D-STAT. Nuclear extracts prepared from S2 cells were treated as indicated. Transfections were with 20 μg of *D-Stat* alone or with 10 μg of *D-Stat* plus two different dosages of *hop* (5 μg in lane 5 or 10 μg in lane 6), which was incubated with ^{32}P-labeled ODBS probe (for optimum D-STAT-binding site) and subjected to gel mobility shift reaction.

(B) Tyrosine mutant D-STAT protein does not bind DNA. S2 cells were stimulated with vanadate–H_2O_2 mixture for 15 min (lane 2) or to 1 hr (lane 3). D-STAT(YF) is D-STAT with Tyr-704 mutated to Phe-704.

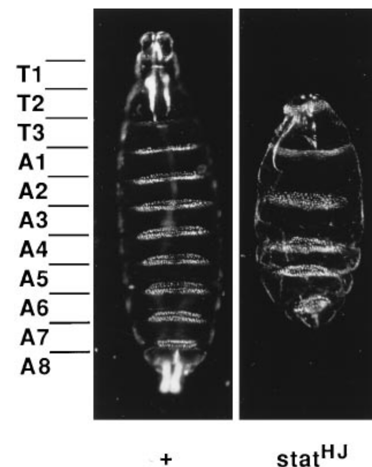


Figure 6. Pair Rule Segmentation Pattern of *stat*^{HJ} Mutants

Examples of wild-type (plus) and homozygous *stat*^{HJ} cuticle preparation are shown. The *stat*^{HJ} embryo exhibits the apparent loss of cuticular structures and naked cuticle from T2 and from the odd-numbered abdominal segments. The segments in the normal pattern are designated to the left.

stat^{HJ}, in the *D-Stat* locus at 92E. A detailed description of the selection of this mutation and its resulting phenotype is given elsewhere (Yan et al., submitted). In summary, *stat*^{HJ} was recovered in a chemical mutagenesis screen for second site suppressors of *hop*$^{Tum-l}$, a dominant mutation in the HOP JAK (Hanratty and Dearolf, 1993). The mutant *stat*^{HJ} locus contains a nucleotide substitution within the first intron, resulting in a reduction in the number of correctly spliced transcripts. In addition, the incorrectly spliced mRNA encodes a truncated 41 amino acid polypeptide that has a dominant-negative effect on transcriptional activation by the wild-type *Stat* cDNA in tissue culture cell assays. The *stat*^{HJ} mutation causes reduced viability of homozygotes, and the mutants exhibit patterning defects that include the formation of ectopic wing veins and abnormalities in segmentation. The majority of *stat*^{HJ} homozygotes have larval segmentation defects similar to those seen in strong recessive *hop* alleles. These defects include the loss of cuticular structures from one or several segments, most frequently the fifth abdominal segment. However, approximately 6% of the *stat*^{HJ} mutants examined had a more extreme phenotype, including a pair rule segmentation pattern (Figure 6).

Binding of D-STAT to *eve* Stripe 3 Promoter Sequences

The second set of experiments that connected D-STAT function to early Drosophila development examined the expression of the pair rule gene *eve*. Genetic experiments indicate that embryos lacking either maternal HOP tyrosine kinase activity (Binari and Perrimon, 1994) or with an abnormal D-STAT content (Figure 6) showed specific cuticular defects, as well as segmental and wing defects. For example, *hop* embryos showed decreased expression in stripes 3 and 5 for the pair rule gene *eve* and in stripe 5 for *run* (Binari and Perrimon, 1994); this expression pattern might therefore require D-STAT. The stripe 3 *eve* enhancer (a 500 bp region) lies between 3.4

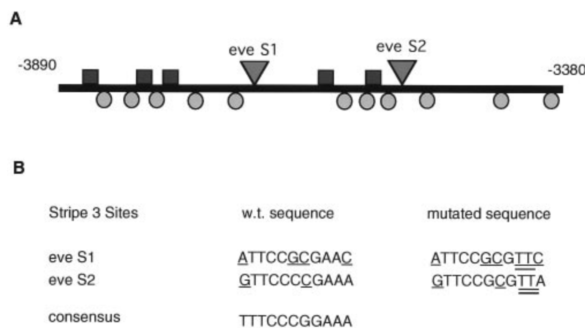


Figure 7. Sequences Binding Activated D-STAT Exist in the *eve* Promoter Region

(A) A schematic representation of the 500 bp enhancer that directs expression of EVE at the position of stripe 3. The positions of D-STAT-binding sites (triangles) are shown in relation to binding sites for the gap proteins, HB (circles) and KNI (squares), which are involved in repression to form the borders of the stripe in vivo (Small et al., 1996).

(B) Sequences of the sites in the stripe 3 enhancer that bind activated D-STAT protein in vitro. Mismatches with the consensus sequence are underlined. The sequences of mutated sites used in the P-element transformation experiments (see Figure 8) are shown at the right. Base substitutions from the wild-type sequence that abolish in vitro binding of activated D-STAT are double underlined.

and 3.9 kb upstream of the *eve* transcription start site (Small et al., 1996; Figure 7A) and directs reporter gene expression at the position of stripe 3 as well as expression of a weak stripe 7. Therefore, we searched the 500 bp stripe 3 enhancer for sequences that resemble the consensus D-STAT-binding site. Two separate sequences (S1 and S2) that contained the $TTCN_3GAA$ motif were identified (Figure 7A) and subsequently tested by EMSA for their ability to bind specifically to Schneider cell extracts that contained activated D-STAT as a result of *hop* coexpression. Both oligonucleotides representing the S1 and S2 sites were shown to bind to the activated D-STAT (only labeled S2 is shown in Figure 7), with S2 showing somewhat stronger competition than S1 in a competition assay (data not shown).

In Vivo Role of D-STAT-Binding Sites

We next performed a series of P-element transformation experiments to determine whether the binding sites identified by homology and in vitro binding experiments were required for the correct expression of the stripe 3 response in vivo. Four lacZ reporter gene constructs were prepared using either the wild-type stripe 3 enhancer or enhancers that contained mutations either individually in S1 or S2 or in both binding sites (Figure 7). Embryos from transgenic fly lines containing these constructs were then assayed for lacZ RNA expression by in situ hybridization. The lacZ expression driven by these mutated enhancers was compared with that directed by the wild-type stripe 3 enhancer. To control for copy number and levels of stripe 3 expression, lines carrying the wild-type and mutated stripe 3 enhancers were crossed with another transgenic line that expresses lacZ at the position of stripe 2 (Small et al., 1992). Thus, the embryos shown in these experiments each contain a single copy of the stripe 2 lacZ construct and a single copy of the stripe 3 construct to be tested.

The wild-type stripe 3 enhancer directed expression of a stripe of lacZ in position 3 that is similar in intensity to stripe 2 (Figure 8A). In addition to the major stripe in the embryos containing either the stripe 2 or stripe 3 enhancers, there were in each case subsidiary stripes. An anterior stripe that was weaker than stripe 2 was observed in the strain carrying the stripe 2 enhancer. This stripe does not correspond to a known structure and is thought to be dependent on vector sequences outside the *eve* enhancer lacZ region (S. S., unpublished data). Strains carrying the wild-type stripe 3 enhancer exhibited a strong stripe 3 expression as well as a weaker stripe 7 expression. Mutating the lower affinity D-STAT-binding site (S1 site) in the stripe 3 enhancer resulted in a dramatic reduction of stripe 3 expression levels, but both stripe 3 and 7 were still visible (Figure 8B). When the higher affinity D-STAT site (S2 site) was mutated, both stripe 3 and 7 were even more drastically reduced (Figure 8C). Mutating both sites completely abolished both stripe 3 and stripe 7 (Figure 8D). These results strongly suggest that the identified D-STAT-binding sites are critical for stripe 3 activation. The reduction of stripe 7 expression was not expected, since the formation of *eve* stripe 7 was not significantly affected in *hop* mutant embryos (Binari and Perrimon, 1994). However, it is possible that activation of D-STAT in the region of stripe 7 is independent of HOP.

eve Expression in $stat^{HJ}$ Mutants

We next tested whether a stock homozygous for the mutant $stat^{HJ}$ allele, which produces a pair rule abnormality in some of the embryos (R. Y. and C. R. D., unpublished data), expressed *eve* stripes normally. Using anti-EVE antiserum, approximately 50% of the embryos collected from a homozygous (early- to mid-cycle 14) $stat^{HJ}$ stock showed some disruption of the normal EVE pattern. However, we detected a significant degree of variability in the disrupted patterns. For example, during the period when the stripes are being initially activated, there was a significant delay in the expression of stripes 3 and 5 in about 30% of the embryos (compare Figures 9A and 9B). Later in cycle 14 (about 2.5–3 hr postfertilization), we detected a number of embryos (10%–15%) that contained globally disrupted patterns composed of 5 stripes (Figures 9D and 9F). It is very difficult to determine which stripes are missing in these embryos because of the aberrant spacing of the stripes that are present. Another significant fraction (10%–15%) of the embryos showed aberrantly spaced seven-stripe patterns (data not shown). Such variability is not surprising given the incomplete penetrance of the mutation in the $stat^{HJ}$ stock. However, these results strongly suggest that wild-type D-STAT function is necessary for normal EVE stripe patterning and, hence, for normal development.

Discussion

The pervasive utilization of the JAKs and STAT proteins by extracellular ligand–signaling proteins in mammalian cells (Schindler and Darnell, 1995), plus the existence of a JAK in Drosophila (Binari and Perrimon, 1994), made

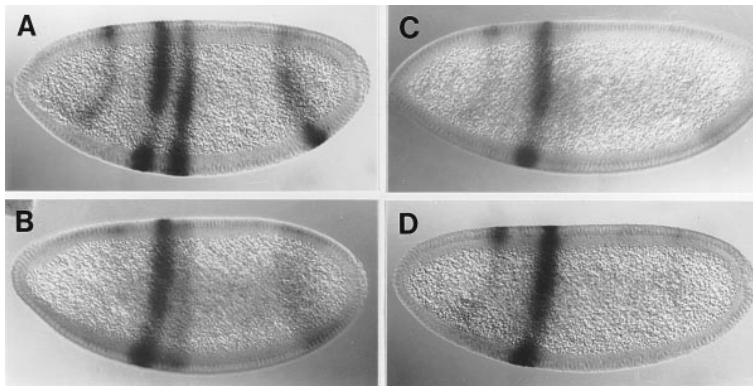


Figure 8. Point Mutations in D-STAT-Binding Sites Abolish Stripe 3 Expression In Vivo

The embryos shown here carry a single copy of a reporter gene construct containing the wild-type stripe 3 enhancer (A) or enhancers containing mutations in the *eve* S1 site (B), *eve* S2 site (C), or in both binding sites (D). All embryos also contain a single copy of a stripe 2 reporter construct to control for expression levels directed by the stripe 3 enhancers. Embryos are at the cellular blastoderm stage and oriented with anterior to the left and dorsal up. Expression of the lacZ reporter genes was detected by histochemical staining after in situ hybridization. All embryos shown in this figure were collected, stained, and photographed in parallel, permitting the direct comparison of expression levels.

it likely that an adequate search would uncover a STAT protein in Drosophila. Indeed, a STAT molecule with considerable sequence similarity to the mammalian proteins was found.

The similarity of the cloned D-STAT sequence with mammalian STAT protein sequence suggested strongly that the Drosophila protein should be a tyrosine-phosphorylated DNA-binding protein. The biochemical experiments reported here strongly reinforce that conclusion; the protein can be phosphorylated on a single tyrosine by two artificial approaches, coexpression with D-STAT and HOP, a JAK, or inhibition of tyrosine phosphatases with vanadate. The implication is that one natural route of tyrosine phosphorylation of D-STAT is by an activated JAK, perhaps HOP. However, the ligand for such an event remains unknown in Drosophila. In fact, Drosophila receptors similar to cytokine receptors that lack intrinsic tyrosine kinase intracytoplasmic domains and activate STATs have not been identified, nor

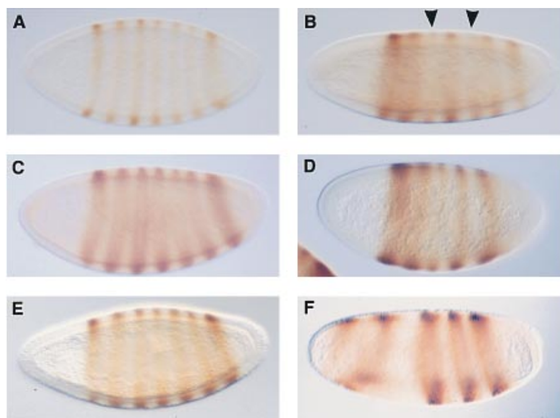


Figure 9. EVE Expression in $stat^{HJ}$ Mutants

Homozygous embryos from the $stat^{HJ}$ stock were examined by antibody staining with antiserum against EVE protein 2–3 hrs after eggs were laid. Most (>70%) of the embryos gave the classic seven-stripe pattern (A, C, and E). A variety of aberrant phenotypes were seen in the remaining embryos. For example, weak or missing stripe 3 was most common (B); also seen were diminished numbers of stripes with poor spreading, so that stripe count was not possible (D), and missing stripe 3 and stripe 7 (E).

have any polypeptide ligands been found in Drosophila that are homologous to the interleukins, growth hormone, or the interferons, examples of ligands that activate the JAK–STAT pathway. Since receptors with intrinsic tyrosine kinases also activate STATs, perhaps such a receptor activates D-STAT.

The DNA-binding site through which the activated STAT might act has a strong resemblance to sites favored by mammalian STATs. The $TTCN_3GAA$ motif is a dyad symmetrical element, implying that the active DNA-binding protein is a dimer. This idea is supported by the finding that the D-STAT–DNA complexes migrate as several different bands in the gel shift analysis. These results suggest that heterodimers may be forming between D-STAT and an endogenous STAT from culture cells. The existence of heterodimers between two mammalian STAT proteins (Stat1 and Stat3) has been demonstrated previously (Zhong et al., 1994b).

That the activated DNA-binding form of D-STAT functions in stimulating transcription in Drosophila is clearly implied, first because the mutant $stat^{HJ}$ that maps at 92E is capable of suppressing the hyperactive JAK in $hop^{Tum\text{-}l}$ and by itself causes a pair rule phenotype in some of the offspring. Second, the requirement for D-STAT-binding sites in the *eve* stripe 3 enhancer clearly shows the importance of these sites in vivo. Moreover, the $stat^{HJ}$ mutant forms aberrant EVE stripes, again implicating D-STAT in proper EVE parasegment expression.

A final point regarding the mechanism of D-STAT activation of the *eve* promoter in stripe 3 concerns the presence of two D-STAT sites in the *eve* stripe 3 promoter, both of which are required for full EVE stripe expression. This result implies a possible interaction between the bound D-STAT (presumably between bound dimers) or a greater than additive effect of the STATs in binding some other transcription factor(s). The former seems likely based on a recent report that two Stat1 homodimers can bind synergistically to two adjacent DNA-binding sites that are individually very weak (Guyer et al., 1995). Furthermore, recent experiments suggest that purified, tyrosine-phosphorylated STAT may form a stable tetramer in vitro (U. Vinkemeier and J. E. D., unpublished data). Thus, it is possible that the binding of a STAT tetramer to two adjacent sites may play a role in

assembling multiprotein enhancer complexes (Tjian and Maniatis, 1994).

The effect of STAT protein on expression of discrete stripes of a pair rule gene raises an interesting question about the cellular structure of the early Drosophila embryo. Cellularization of the syncytial blastoderm of Drosophila is not complete at the time of *eve* expression. Thus, signaling of individual cells is probably not involved in activating the HOP–D-STAT pathway in the precellular blastoderm. However, if a receptor-mediated event is responsible for activating this pathway, it could originate in the perivitelline space between the cytoplasmic membrane that encloses the embryo and the vitelline membrane. It has been shown previously that localized signals from the perivitelline space are important for terminal and dorsal–ventral patterning (reviewed by St Johnston and Nüsslein-Volhard, 1992). Perhaps an as yet unidentified receptor-mediated phosphorylation cascade might be involved in activating D-STAT. Recent microscopic examination of fertilized eggs and syncytial blastoderm stages in Drosophila development with phosphotyrosine antibodies shows tyrosine phosphates appearing at the membrane by 2.5 hr of development (approximately coincident with *eve* expression). The invaginations of membrane around the peripherally placed nuclei also stain brightly for tyrosine phosphoproteins (Loncar and Singer, 1995). Thus, it seems very likely that ligand-activated surface events in the JAK–STAT pathway do occur at this time. If this is true, the mechanism for restricted effect of JAK–STAT activation might be caused by a specific distribution of either ligand or receptor for D-STAT to contribute to some, but not all, stripe formation.

Alternatively, the signal need not be spatially restricted. For example, the HOP–D-STAT pathway might be activated throughout the developing embryo, but activation of specific *eve* stripes would result from decreased levels of repressors that repress the stripe 3 enhancer only at the level of stripe 3 (and possibly stripe 7). As discussed earlier, a modular 500 bp enhancer controls the expression of stripe 3 and a weaker stripe 7. Genetic analysis has shown that the expression mediated by this enhancer can be expanded throughout the embryo by systematic removal of repressors that suppress stripe formation in different regions of the embryo (Small et al., 1996). In fact, removing the right combinations of repressors allows this enhancer to be active throughout the embryo, consistent with the possibility that the activator might be ubiquitously expressed. The repressor proteins HB and KNI, which are expressed in domains that define the anterior and posterior borders of stripe 3, both bind in vitro to multiple sites within the stripe 3 enhancer (Figure 7). Some repressor sites are in very close proximity to the D-STAT-binding site (in particular DBS2), suggesting that local interactions between proteins bound to the enhancer are responsible for generating the pattern. This mechanism is very similar to that proposed for the regulation of *eve* stripe 2 (Small et al., 1992). The enhancer that drives stripe 2 expression can be activated by the broadly distributed activators BCD and HB, but the borders of the stripe are set by the localized repressors GT and KR.

In conclusion, the larger implication of the experiments of the Perrimon group (Binari and Perrimon, 1994) and our own experiments is that the JAK–STAT signaling system does exist in Drosophila and that it is used in the regulation of cell fate in early development.

Experimental Procedures

Molecular Cloning of *D-Stat*

A Drosophila embryonic cDNA library (0–24 hr; a gift from Dr. Simon Kidd at the Rockefeller University) was used as template DNA for PCR amplification with degenerate primers based on sequences within the mammalian SH2 domain.

Preparation of DGG-Labeled Riboprobes

The plasmid 171x used for RNA probe preparation contains the *D-Stat* cDNA fragment from 792 to 1314 inserted into pBluescript SK(−) vector at the XhoI site. RNA probes for both orientations labeled with DGG–UTP were synthesized as described by the manufacturer (Stratagene) and purified by LiCl and ethanol precipitation with the addition of carrier tRNA. An RNA probe derived from the plasmid *Hairless* cDNA 2-10 (Bang and Posakony, 1992) was also made. Routinely, an RNA probe was synthesized from 1 μg of linearized plasmid DNA and the purified RNA probe was dissolved in 200 μl of DEPC-treated H_2O and stored at −20°C.

Polytene Squash Hybridization

Salivary glands dissected from third instar larvae were used for squash preparation of polytene chromosomes. Each slide was extensively washed with 2× SSC at 68°C for 30 min, dehydrated by soaking for 5 min in 70% ethanol and for another 5 min in 95% ethanol, and air dried. The squash was denatured with 0.07 M NaOH for 3 min and washed three times with 2× SSC for 5 min, followed by dehydration as above. Each squash was hybridized at 37°C overnight in 10 μl of hybridization solution containing 50% formamide, 3× SSC, 100 ng of salmon sperm DNA, and 2 μl of either sense or antisense labeled riboprobe. The excess probe was washed off with 2× SSC at 37°C for 30 min, PBS containing 0.1% Tween 20 (PBT) at room temperature for 5 min, and PBS briefly. The squash was then incubated with alkaline phosphatase–conjugated anti-DGG antibody at 37°C for 1 hr. After being washed with 2× SSC, PBT, and PBS as described above, hybridization was visualized with NBT–BCIP staining solution. Chromosomes were then stained with 5% Giemsa in PBS.

In Situ Hybridization and Antibody Staining

In situ hybridization and antibody staining of Drosophila embryos were performed as described previously (Simpson-Brose et al., 1994) using an antisense RNA probe synthesized from plasmid 171x, with the sense probe used as a negative control. In the case of double staining, monoclonal antibody against Engrailed protein was incubated with those embryos that were first hybridized with *D-Stat* riboprobes and then fixed in 70% ethanol for at least 2 hr.

Antibody Production

The glutathione S-transferase (GST) fusion protein included the first 113 amino acids from D-STAT (Sambrook et al., 1989) and was used (400 mg) to immunize a female New Zealand rabbit. The antiserum was collected and tested by Western blot analysis after each boost. Since the produced antibody reacted with quite a few Drosophila proteins, both the GST polypeptide and the GST–D-STAT (1–113) fusion proteins were coupled to the CNBr-activated agarose beads (Sigma). The serum (10 ml) pooled after the third boost was loaded onto the column packed with 1 ml of GST–D-Stat (1–113)–agarose beads. The column was extensively washed with PBS, and the bound antibody was eluted with elution buffer containing 200 mM glycine and 1 mM EDTA (pH 2.8). The eluate was neutralized immediately with one-tenth volume of 1 M Tris (pH 7.0) and loaded onto a GST–agarose column to remove any antibody that reacted with GST epitopes. The flowthrough was concentrated by centricon (Amicon) to 1 mg/ml. This antiserum was used in Western blots.

Tyrosine Phosphorylation Analysis of D-STAT in Cultured Cells

The entire coding region of *D-Stat* including the FLAG epitope added at the C-terminus was amplified by PCR with *Vent* polymerase (NEB) and subcloned into Schneider cell expression vector pPAC5C-PL under the control of actin 5C promoter. The tyrosine mutant construct (Tyr-704→Phe) was made by PCR with Taq polymerase (Boehringer Mannheim) followed by sequencing verification and subcloned into the vector pPAC the same way as the wild-type construct. The coding region of *hop* was inserted into the pPAC5C-PL vector at a NotI site. Schneider 2 (S2) cells were cultured in Shield and Sang M3 insect medium supplemented with 10% fetal bovine serum. Transient transfection of each 10 cm dish with a total of 20 μg of DNA was performed by the calcium phosphate precipitate method. After being transfected for 48–60 hr, the cells were harvested and then used for preparation of cytosolic and nuclear extracts. Vanadate treatment consisted of a 15 min exposure to 1 mM sodium orthovanadate and 2 mM H_2O_2 for 15 min prior to extraction (Larner et al., 1993; Zhong et al., 1994a). For immunoprecipitation experiments, all the cytosolic extracts plus 90% of the nuclear extracts were pooled and reacted with monoclonal anti-FLAG antibody (M2Ab)–agarose beads (IBI Kodak) for 2 hr. The unbound proteins were washed away with PBS with 0.1% Tween 20. The bound proteins were eluted from the bead by heating at 90°C for 3 min in 1× SDS–polyacrylamide gel loading buffer, resolved with 7% SDS–PAGE, and transferred to a nitrocellulose membrane. Immunoblots were first reacted with Py20 antibody (Signal Transduction Laboratory) and subsequently reprobed with M2Ab and anti–D-STAT N-terminal antibody after each stripping.

Optimum DNA-Binding Site Selection for an Activated D-STAT

Oligonucleotides with 26 random sites flanked by known PCR primers of 25 bp were made as described previously (Tanaka et al., 1994). Binding site selection was performed according to the method of Pollock and Treisman (1990). Nuclear extracts prepared from *D-Stat*-transfected S2 cells stimulated with vanadate–H_2O_2 mixture for 15 min were used to provide DNA-binding factors. M2Ab precipitation was used to enrich oligonucleotides bound to the D-STAT protein. After the fifth round of selection, the copurified DNA was amplified with PCR and used for gel mobility shift assay (Shuai et al., 1992). Two faint shifted bands (equivalent to band A and B in Figure 3; data not shown) were observed. Those two bands were excised, and DNA was extracted, reamplified, and analyzed by the gel mobility shift assay. After a final gel shift, PCR products from the DNA–protein complexes were amplified, purified, and subcloned into pBluescript SK(−) (Stratagene), and 30 independent clones were sequenced.

EMSA

Gel mobility shift assays were carried out as described previously (Levy et al., 1989). All the oligonucleotide probes were labeled with [^{32}P]dNTP by filling in the protruding end of ACTG, and 1 ng of each labeled probe was reacted with activated D-STAT in each gel mobility shift reaction. The oligonucleotide ODBS (for optimum D-STAT-binding site) with the top strand sequence 5′-GGATTTTTCC CGGAAATGGTC-3′ was synthesized based on the results of the binding site selection. Two oligonucleotides, S1, 5′-GACTCCCATTC CGCGAACCGG-3′, and S2, 5′-CGATCCGGTTCGCGGAATGGG-3′, were prepared from sequences within the *eve* stripe 3 enhancer region (Small et al., 1996).

DNA Constructs

The mutagenesis of the D-STAT sites in the *eve* stripe 3 enhancer was carried out using PCR on an ∼540 bp BamHI–XhoI fragment that contained the 500 bp stripe 3 enhancer fused to the *eve* basal promoter. This fragment was cloned into BamHI–XhoI-cut CaSpeR *eve*–lacZ vector, which contains the 100 bp untranslated *eve* leader and the first 22 codons of the *eve* coding region fused in-frame to the lacZ coding sequence at codon Y5 (Lawrence et al., 1987). The mutations were verified by sequence analysis. The CaSpeR vector contains a mini-*white* gene for selection of transformants (Pirrotta et al., 1985).

Embryonic Cuticle Preparations

These were performed according to the method of Wieschaus and Nüsslein-Volhard (1986).

P-Element Transformation Assays

Seven independent transgenic lines carrying the wild-type stripe 3 lacZ constructs were previously generated (Small et al., 1996). For this study, we used standard microinjection techniques (Spradling, 1986) to generate four, three, and six independent lines for the constructs stripe 3 mSTAT1, mSTAT2, and mSTAT1+2, respectively. Embryos collected from these lines were assayed for lacZ expression by in situ hybridization using an antisense lacZ RNA probe as previously described (Jiang et al., 1992).

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During the preparation of this manuscript, we learned that Dr. Norbert Perrimon and colleagues have recovered a P-element insertion in the *D-Stat* gene that completely prevents *D-STAT* mRNA formation.

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