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Genetics

A JAK–STAT pathway regulates wing vein formation in *Drosophila*

(*stat* mutation/pattern formation)

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ABSTRACT We present evidence that the JAK–STAT signal transduction pathway regulates multiple developmental processes in *Drosophila*. We screened for second-site mutations that suppress the phenotype of the hyperactive $hop^{Tum\text{-}l}$ Jak kinase, and recovered a mutation that meiotically maps to the known chromosomal position of *D-Stat*, a *Drosophila stat* gene. This hypomorphic mutation, termed $stat^{HJ}$, contains a nucleotide substitution in the first *D-Stat* intron, resulting in a reduction in the number of correctly processed transcripts. Further, the abnormally processed mRNA encodes a truncated protein that has a dominant negative effect on transcriptional activation by the wild-type cDNA in cell culture. $stat^{HJ}$ mutants exhibit patterning defects that include the formation of ectopic wing veins, similar to those seen in mutants of the epidermal growth factor/receptor pathway. Abnormalities in embryonic and adult segmentation and in tracheal development were also observed. The $hop^{Tum\text{-}l}$ and $stat^{HJ}$ mutations can partially compensate for each other genetically, and Hop overexpression can increase D-Stat transcriptional activity *in vitro*, indicating that the gene products interact in a common regulatory pathway.

Members of the JAK family of nonreceptor tyrosine kinases mediate the cellular response to a large number of cytokines and growth factors in mammalian cells (for reviews, see refs. 1 and 2). Most often, binding of the appropriate ligand to its cell surface receptor leads to activation of the Jak kinase/receptor complex. This in turn results in the tyrosine phosphorylation of one or more cytoplasmic STAT (*s*ignal *t*ransducer and *a*ctivator of *t*ranscription) proteins, which translocate to the nucleus and regulate the transcription of ligand-inducible genes.

The fruit fly *Drosophila melanogaster* offers an excellent genetic system to fully elucidate the components of JAK–STAT signaling pathways and to determine the developmental roles of Jak and Stat proteins. There is one known JAK kinase in *Drosophila*, encoded by the *hopscotch* (*hop*) locus (3). Embryos lacking maternal and zygotic *hop* products die before hatching and frequently have defects in segmentation (4). Mutants lacking only the zygotic component of *hop* expression are larval or pupal lethal. In the most severe mutants, the diploid imaginal precursor cells appear unable to respond to proliferative signals in the larvae, so that the diploid tissues in larvae are severely reduced in size (4). In addition, two dominant alleles, $hop^{Tum\text{-}l}$ (5) and hop^{T42} (H.L., W. P. Hanratty, and C.R.D., unpublished data) have been identified. These dominant mutations cause several defects, including a fruit fly "leukemia," the overproliferation, premature differentiation, and aggregation of hemocytes, the formation of melanotic masses of hemocytes and gut tissue, and the reduced viability of the mutant stock (6).

Recently, we and others have identified a *Drosophila* Stat gene (*D-Stat*) (7, 8). *hop* and *D-stat* genetically interact to regulate embryonic segmentation, in part by influencing the transcription levels of the *even-skipped* (*eve*) segmentation gene. Phosphorylated D-Stat protein binds to a TTTCCCGGAAA consensus DNA recognition site that is similar to mammalian STAT binding sites.

In this paper, we demonstrate that JAK–STAT pathways regulate multiple developmental processes in *Drosophila*, including the formation of ectopic adult wing veins. We report the generation and characterization of a hypomorphic *D-Stat* mutation that also can behave as a partial dominant negative. We also document that *hop* overexpression can activate D-Stat activity in tissue culture cells. Our results demonstrate the utility of *Drosophila* genetic screens for dissecting Jak kinase regulatory pathways and for determining the role of these signaling networks in pattern formation.

MATERIALS AND METHODS

Fly Culture and Crosses. Flies were grown on standard cornmeal/molasses/agar/yeast medium supplemented with Tegosept (Sigma). Cultures were kept at 65–70% relative humidity, and at the temperatures indicated. Common stocks are described in ref. 9. The $stat^{HJ}$ third chromosome originally recovered contained additional mutations, including a (Y;3) reciprocal translocation at chromosome position 69D and an unmapped recessive lethal mutation. To remove these additional mutations and to meiotically map the $stat^{HJ}$ mutation, we used C(1)M4 *y; ru h th st cu sr e ca* attached-X females.

Characterization of the $stat^{HJ}$ Phenotype. The $stat^{HJ}$ stock used in these experiments carries the markers *ry* and *e*. Control stocks used were Oregon-R and *ry* $stat^{+}e$. To quantitate the suppression of $hop^{Tum\text{-}l}$ lethality, *y v* $hop^{Tum\text{-}l}$/Basc females were mated to *ry* $stat^{HJ}$ *e* or to control males at 25°C. Parents were transferred daily to new bottles, with the progeny shifted to 29°C. Care was taken to avoid overcrowding, which inhibits the recovery of $hop^{Tum\text{-}l}$ hemizygotes. Embryonic cuticle preparations were performed as described (10).

Reverse Transcription–PCR. Isolation of total RNA from $stat^{HJ}$ larvae was performed as described (11). Polyadenylylated mRNA was further extracted with QuickPrep Micro mRNA purification kit (Pharmacia) and used for standard first strand cDNA synthesis with Moloney murine leukemia virus reverse transcriptase (GIBCO/BRL) with the following primers: primer N, 1680-CTAGAGGCGTTTTGAATC-1663; primer M, 1980-GTAACGCGGTCGGTAATC-1963; and primer C, 2760-AGCGACACACGTTCAGGA-2743. The following pairs of primers were used for the nested PCR amplication: primer 1, 403-ATCCATCGGGTAGAGCAA-420; primer 2, 1560-GGTGGATTGTTGTGAATG-1543; primer 3, 1181-ATGTACTACGAATCGCTG-1198; primer 4, 1923-CAAGTAATGGTAGCCCAA-1905; primer 5, 1876-TATT-

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Abbreviation: CAT, chloramphenicol acetyl transferase.

GTGCACGGCAACCA-1893; primer 6, 2714-TTCTATAGT-GGTTATAGT-2697; and primer 7, 591-CGCTCTAACT-GATCGGTA-574.

Chloramphenicol Acetyltransferase (CAT) Activity Assay. The entire coding region of D-Stat plus a FLAG epitope at the C terminus was amplified by PCR with *Vent* polymerase (New England Biolabs), and subcloned into Schneider cell expression vector pPAC5C-PL. The coding region of *hopscotch* was inserted into the pPAC5C-PL vector at its *Not*I site. The coding region from the $stat^{HJ}$ D-Stat with the first intron included was inserted into pPAC5C-PL at the *Bam*HI and *Xba*I sites. Two copies of the optimum D-Stat binding site ODBS (7) were inserted into the pD-33-CAT vector at the *Bam*HI site, producing the 2×DBS construct. Transfection of various DNA mixtures in Schneider cells was performed by the calcium phosphate precipitate method (12). At 60 h posttransfection, cells were transferred to a 15-ml Falcon tube, washed with PBS, and collected by centrifugation. The cell pellet was suspended in 0.25 M Tris, pH 7.7, and lysed by being frozen on dry ice and thawed quickly in a 37°C water bath for three cycles. The lysate was centrifugated at 4°C for 5 min and the supernatant was transferred to a fresh tube for determination of protein concentration and the following CAT assay. The CAT reactions were carried out with the protocal described (13) and radioactivity was measured by PhosphorImager (Molecular Dynamics).

RESULTS

Generation of a *stat* Mutation. To identify other *Drosophila* genes that could participate in *hop* (Jak kinase) signal transduction pathways, we devised a mutagenesis screen for dominant autosomal suppressors of the $hop^{Tum\text{-}l}$ phenotype. $hop^{Tum\text{-}l}$ is a dominant, temperature-sensitive mutation (5). The mutant protein contains a glycine to glutamic acid substitution (14) that causes the Hop JAK kinase to be hyperphosphorylated on tyrosine (15) and hyperactive (H.L. and C.R.D., unpublished observations). At high culture temperatures, the mutation causes several defects, including abnormalities in hemocyte proliferation and differentiation, the melanization of hematopoietic and gut tissue, and the lethality of hemizygous males (6).

To conduct the mutagenesis screen, a wild-type Oregon R stock was first made isogenic for both the second and third chromosomes. Then males from this stock were mutagenized with the chemical ethylmethane sulfonate (EMS), using the adult feeding method (16). These males were mated to *y v* $hop^{Tum\text{-}l}$ *m*/Basc females, which are heterozygous for $hop^{Tum\text{-}l}$ on the X chromosome. Following one day of oviposition at 25°C, the progeny were raised at the tumor-permissive temperature of 29°C. We then screened the surviving adults for $hop^{Tum\text{-}l}$ males, which might possess dominant, second-site suppressor mutations. To facilitate the efficiency of the mutagenesis screen, the $hop^{Tum\text{-}l}$ stock used for the screen had undergone several months of selection for a strong $hop^{Tum\text{-}l}$ phenotype. In this selected stock, hemizygous $hop^{Tum\text{-}l}$ male escapers are recovered at a rate of less than 0.1% for the conditions used (C.R.D., unpublished observations). All surviving F_1 adult males hemizygous for $hop^{Tum\text{-}l}$ were individually remated to *y v* $hop^{Tum\text{-}l}$ *m*/Basc females and tested for the presence of a suppressor mutation. Out of ≈30,000 potentially mutagenized autosomes examined, 14 $hop^{Tum\text{-}l}$ males were recovered. Five of these males transmitted an autosomal suppressor mutation to their progeny that allowed 20% or more of the expected number of $hop^{Tum\text{-}l}$ males to survive at 29°C.

One of these supressor mutations was a lesion in the *D-Stat* gene (see below) and has been named $stat^{HiJak}$ ($stat^{HJ}$; so named because the mutation interacts with the hyperactive JAK kinase). The mutation was mapped by meiotic recombination to 3-68.9, the flanking markers being *stripe* (3-62.0, 90D-F) and *ebony* (3-70.7, 93D) (9). This mapping location is consistent with the *D-Stat* salivary gland chromosome position at 92E (7, 8). We found that one copy of $stat^{HJ}$ significantly suppressed $hop^{Tum\text{-}l}$ lethality (Table 1). At 29°C, more than half of the expected number of $hop^{Tum\text{-}l}$; $stat^{HJ}$/+ males survived, compared with 4–5% of the $hop^{Tum\text{-}l}$; $stat^{+}$ males. The dominant $hop^{Tum\text{-}l}$ phenotype was not completely eliminated in a $stat^{HJ}$ background, however, as some hematopoietic abnormalities and melanotic mass formation were still observed.

Phenotypic Analysis of $stat^{HJ}$. We then examined the $stat^{HJ}$ phenotype in a background that was wild type for the *hop* gene. We found that the $stat^{HJ}$ mutation affects multiple developmental processes (Figs. 1 and 2). The mutant phenotype is strongest in $stat^{HJ}$ homozygotes, but also occurs in heterozygotes. Further, the mutation is not temperature sensitive, in contrast to $hop^{Tum\text{-}l}$ (Table 2). First, we determined that the viability of $stat^{HJ}$ mutants is decreased. We found that 1401/2028 (69%) of homozygous $stat^{HJ}$ larvae hatched and survived to adulthood compared with 1497/1669 (90%) for controls with a similar genetic background (data not shown).

We also observed that the wing blades of viable $stat^{HJ}$ adults have ectopic vein formation in the posterior wing blade compartment. These ectopic longitudinal veins appeared as a partial vein connected to the posterior crossvein, and/or as a partial vein located posteriorly to vein L5 (Fig. 1*B*). Greater than 40% of the $stat^{HJ}$ heterozygotes examined had at least one abnormal wing, whereas almost every homozygote was affected (Table 2).

The ectopic wing vein formation in $stat^{HJ}$ mutants could be suppressed by changes in *hop* JAK kinase activity or expression level (Table 2). The $hop^{Tum\text{-}l}$ mutation drastically reduced the severity of the phenotype in $stat^{HJ}$ mutants. The extent of this suppression was temperature-dependent, consistent with the temperature sensitivity of the $hop^{Tum\text{-}l}$ mutation. The $stat^{HJ}$ wing vein phenotype was also suppressed by one copy of the transgenic Act/T-r7 chromosome, which constitutively overexpresses hop^{+} transcripts (14). These results demonstrated that the formation of ectopic wing veins was sensitive to the activities of both the *hop* Jak kinase and $stat^{HJ}$.

To verify that the ectopic wing veins observed in $stat^{HJ}$ mutants were caused by mutation in the *D-stat* locus, we tested for the rescue of the mutant phenotype in transgenic flies. We generated a heat shock promoter/$stat^{+}$ cDNA construct and obtained four independent transgenic strains by P-element transformation. We mated *ry* $stat^{HJ}$ *e* homozygous females to a stock homozygous for a transformant on the second chromosome. Eggs were collected for 24 h, then subjected to a 30 min heat pulse of 37°C on a daily basis throughout the larval and pupal stages. We observed that the overexpression of wild-type *stat* cDNA can partially rescue the heterozygous $stat^{HJ}$ wing vein phenotype (Table 2). Only 10% of the $stat^{HJ}$ mutants containing the transgenic chromosome had one or more wings with ectopic veins. In contrast, 35% of the control mutants lacking the heat shock/$stat^{+}$ construct had an abnormal wing. Therefore, the ectopic vein formation seen in $stat^{HJ}$ mutants is indeed caused by an abnormality in the *D-stat* locus.

Table 1. The suppression of $hop^{Tum\text{-}l}$ lethality at 29°C by the $stat^{HJ}$ mutation

Parental cross (females × males)	Total progeny recovered	Males	
		Basc	$hop^{Tum\text{-}l}$
y v $hop^{Tum\text{-}l}$/*Basc* × *Oregon R*	1648	454	24 (5%)
y v $hop^{Tum\text{-}l}$/*Basc* × *ry* $stat^{+}$ *e*	1412	365	14 (4%)
y v $hop^{Tum\text{-}l}$/*Basc* × *ry* $stat^{HJ}$ *e*	1301	269	143 (53%)

The numbers given include those adult males able to completely eclose from the pupal case.

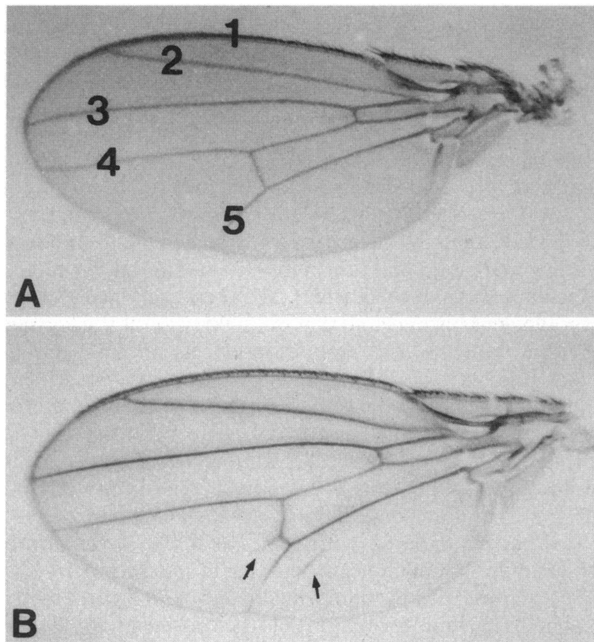

FIG. 1. Wing vein abnormalities in *stat*^{HJ} mutants. (*A*) A wild-type wing blade. The longitudinal veins are indicated. (*B*) A *stat*^{HJ} wing blade. The ectopic longitudinal veins are indicated by arrows.

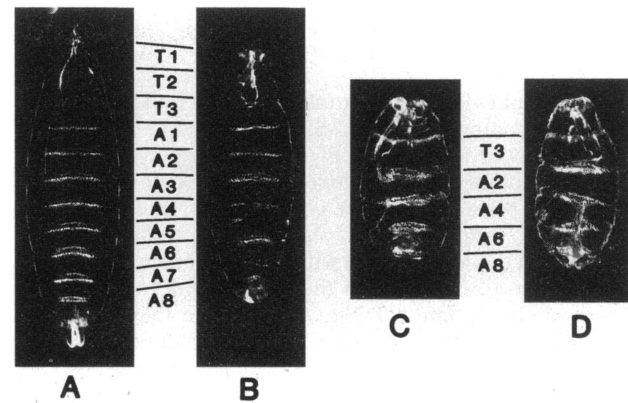

FIG. 2. Examples of *stat*^{HJ} embryonic cuticular defects. (*A*) A wild-type cuticle. The thoracic and abdominal segments are indicated. (*B*) A mild *stat*^{HJ} phenotype. The A5 and A7 denticle belts, as well as filzkörper material, are partially deleted. (*C*) A pair-rule *stat*^{HJ} phenotype exhibiting the apparent loss of cuticular structures from T2 and from the odd-numbered abdominal segments. (*D*) A pair-rule *stat*^{HJ} cuticle with partial fusion of the presumed A4 and A6 segments. The mutant cuticles are frequently more difficult to spread completely, and in *C* and *D* the filzkörpers and posterior spiracles are present underneath the visible denticle belts.

We also observed segmentation defects in *stat*^{HJ} larval cuticles (Fig. 2) (7), the absence or incomplete fusion of abdominal cuticular structures in some *stat*^{HJ} adults, and occasional gaps in the dorsal tracheal trunks of *stat*^{HJ} mutant larvae. The larval segmentation defects were consistent with those reported for strong *hop* loss-of-function alleles (4) and with an apparent *stat* null allele (8). We examined 300 cuticles prepared from mutants whose parents were both *stat*^{HJ}/*stat*^{HJ}. Of the cuticles, 53% (158/300) were normal, 20% (61/300) had abnormalites in one segment, and the remaining 27% (81/300) had defects in two or more segments. The most common defect observed was a partial or complete loss of one or two sets of denticle bands (Fig. 2*B*). The 5th abdominal segments were most often affected, followed by the 7th segments. However, defects in A1 and A3 were also common, and occasionally, defects were observed in only an even-numbered abdominal segment. In some individuals, two or three adjacent segments were missing and/or fused, most commonly in the region between abdominal segments 5–8. Approximately 6% of the mutants examined had the most extreme defects. This severe phenotype includes a pair-rule segmentation pattern, in which the denticle belt and naked cuticle from T2 and the odd numbered abdominal segments were deleted (Fig. 2*C*). Additional defects included the partial fusion of remaining segments (Fig. 2*D*), the partial or complete loss of terminal structures, and failure of the head structures to involute.

Sequence Change in *stat*^{HJ}. Using PCR probes from the D-Stat sequence, various segments of D-Stat mRNA were recovered as DNA copies from the wild-type Oregon R strain and from the *stat*^{HJ} strain for sequence analysis (Fig. 3*A*). All of the copied mRNA segments from 403 nucleotides to 2714 at the 3′ end of the mRNA from both strains were of the expected size. Sequence analysis of these segments, including multiple independent analyses of all regions encoding amino acid residues, showed no changes in the predicted amino acid sequence. However, a difference was observed in the PCR bands from the region of the mRNA representing the N terminus of the protein. In addition to the expected PCR band, there was one slightly larger band from the *stat*^{HJ} strain that was not recovered from the wild-type strain (Fig. 3*C*). The sequence of three independent longer clones representing this 5′ region revealed in each case the presence of a 126 nucleotide stretch. This sequence, which is not present in the wild-type clones, contains stop codons in all three frames and would terminate the D-Stat protein product at 41 amino acids. The extraneous sequence was bordered by GT and AG, the sequences that mark an intron. In addition, there was a pyrimidine-rich stretch from 5–18 nucleotides from the 3′ end of the included sequence. The position of this putative intron is in an analogous position to the first intron in mammalian mRNA

Table 2. Quantitation of the *stat*^{HJ} wing vein phenotype

Stock	Temperature, °C	No. of adults examined	No. of adults with one or two wings abnormal	No. of adults with two wings abnormal
stat^{HJ}/*stat*^{HJ}	17	1274	1263 (99%)	1146 (90%)
stat^{HJ}/*stat*^{HJ}	25	1332	1295 (97%)	1224 (92%)
stat^{HJ}/*stat*^{HJ}	29	1024	1016 (99%)	977 (95%)
hop$^{Tum\text{-}l}$; *stat*^{HJ}/*stat*^{HJ}	17	1085	463 (43%)	180 (17%)
hop$^{Tum\text{-}l}$; *stat*^{HJ}/*stat*^{HJ}	25	1255	171 (14%)	9 (1%)
stat^{HJ}/*stat*$^{+}$	25	1026	505 (49%)	84 (8%)
stat^{HJ}/*stat*$^{+}$	29	1013	442 (44%)	115 (11%)
Act-hop^{+}/+; *stat*^{HJ}/*stat*$^{+}$	25	717	89 (12%)	12 (2%)
stat^{HJ}/+	Heat shocked	675	235 (35%)	26 (4%)
stat^{HJ}/+; *HS-stat*$^{+}$/+	Heat shocked	724	71 (10%)	9 (1%)

(17). Thus, it seemed that at least some of the copies of the $stat^{HJ}$ mRNA had retained an intron.

To test this interpretation, we amplified a PCR product from this region of Oregon R (parent strain) genomic DNA and sequenced ≈300 nucleotides. The results verified that the 126 additional nucleotides observed in the copied $stat^{HJ}$ mRNA do indeed represent the intron that divides D-Stat coding exons 1 and 2 in *Drosophila* genomic DNA. There was a single nucleotide change in this wild type genomic sequence when compared to the intronic sequence recovered from $stat^{HJ}$. This was a T to C at position 61 in the intron (Fig. 3*B*). Therefore, there exists in $stat^{HJ}$ transcripts some mRNA that retains an intron separating the first two coding exons.

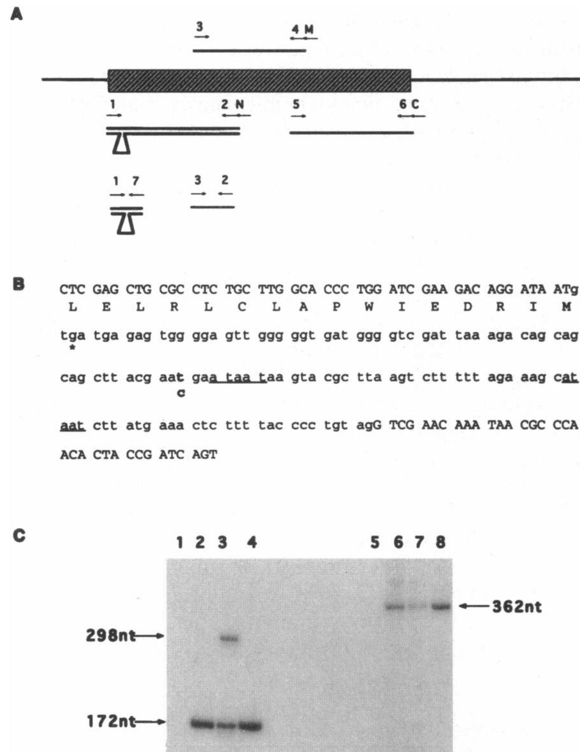

FIG. 3. Identification of nucleotide mutation in $stat^{HJ}$. (*A*) Diagram of RT-PCR scheme used to obtain segments of $stat^{HJ}$ for sequence. The shaded box represents the open reading frame of the D-Stat gene. Each thin line (not proportional to length) represents a primer and the arrow indicates the orientation. Primers M, N, and C were used for synthesis of the first strand cDNA from $stat^{HJ}$ mRNA. Each PCR fragment obtained with the indicated pairs of primers is represented with a solid line. PCR amplification with primers 1 and 2 or 1 and 7 produces a product of the length expected from the wild-type sequence and, in each case, an extra product that contains an intron between the exon 1 and exon 2 in the open reading frame. (*B*) The intron sequence and the partial flanking sequences of exons 1 and 2. The nucleotide sequence including the intron was obtained from a PCR product amplified from the wild-type parental Oregon R strain genomic DNA. The intron is represented with lower case letters and the exon in upper case letters. Amino acid sequence is displayed below the corresponding codon. *, A stop codon. The nucleotide "**t**" was found to be mutated to "**c**" in the $stat^{HJ}$ cDNA. A duplicate stretch sequence of five bases is underlined. (*C*) Semiquantitative PCR. PCR amplification with two pair of primers 1, 7 and 3, 2 was carried out in the standard PCR buffer for *Taq* polymerase (Boeheringer Mannheim) except 2 μCi of [^{32}P]dATP was included in each 100 μl of reaction solution. A 25-cycle PCR amplication used denaturing at 94°C for 1 min, annealing at 52°C for 1 min, and polymerization at 72°C for 15 sec, with template DNA as follows: Lanes 1 and 5, mock template DNA added; lanes 2 and 6, the first strand of cDNA synthesized from Oregon R larvae mRNAs; lanes 3 and 7, the first strand of cDNA derived from $stat^{HJ}$ larvae mRNAs; and lanes 4 and 8, D-Stat cDNA. The expected sizes of the PCR products are marked with arrows.

A semiquantitative PCR analysis of third instar larval mRNA from wild-type and homozygous $stat^{HJ}$ mutants was carried out to detect the frequency of the inclusion of the intron described above (Fig. 3*C*). PCR probes that bracketed the included intron in the cDNA were used. This produced one band at ≈172 nucleotides from copies of wild-type mRNA, but two bands of equal intensity at 172 nucleotides and 298 nucleotides from copies of the $stat^{HJ}$ mRNA. The slower moving band includes the 126 nucleotide intron. This assay indicates that each of the two mRNAs makes up about 50% of the total in the $stat^{HJ}$ strain. The assay was carried out on poly(A)$^+$ RNA from whole third instar larvae. Therefore, it has not yet been determined whether all cells containing D-Stat mRNA have the same distribution of the included intron.

Dominant Negative Effect of the D-Stat N Terminal Protein Fragment. The $stat^{HJ}$ phenotype could be caused by a reduced amount of normal mRNA and/or the production of a truncated protein in the mutants. In the latter case, the truncated $stat^{HJ}$ fragment might interfere with the transcription of genes that normally occurs through activated D-Stat. We therefore tested the possibility that the D-Stat fragment had an effect in a D-Stat dependent transcriptional activation assay (Fig. 4). Since no ligand dependent activation of D-Stat was known, the following approach was taken. Cultured *Drosophila* Schneider cells were transfected with one or more of the following constructs: (*i*) a CAT reporter construct in which transcription of the test gene was driven by two copies of D-Stat binding sites; (*ii*) expression vectors encoding either or both of the wild-type and the mutant $stat^{HJ}$ protein; and (*iii*) an expression vector encoding hop^+ protein. Cells transfected with a reporter vector alone were scored as 1.00. Cells transfected with the CAT vector and *D-Stat* did not increase this basal level of CAT activity. However, cells transfected with *hop* and the CAT construct without the wild-type *D-Stat* did give an enhanced transcriptional signal (Fig. 4), possibly due to endogenous Stat proteins in the Schneider cells. The inclusion of wild-type *D-Stat* with the other vectors produced the strongest transcriptional signal, about 60% greater than the *hop* protein alone. When the $stat^{HJ}$ construct was included in the transfection at a level of DNA equal to that of the D-Stat construct (1:1), there was essentially no effect on the signal. However, increasing the ratio of $stat^{HJ}$ to wild-type DNA to 5:1 or 10:1 during the transfection decreased the D-Stat dependent transcription to a level at or a little below the basal level. Thus, the $stat^{HJ}$

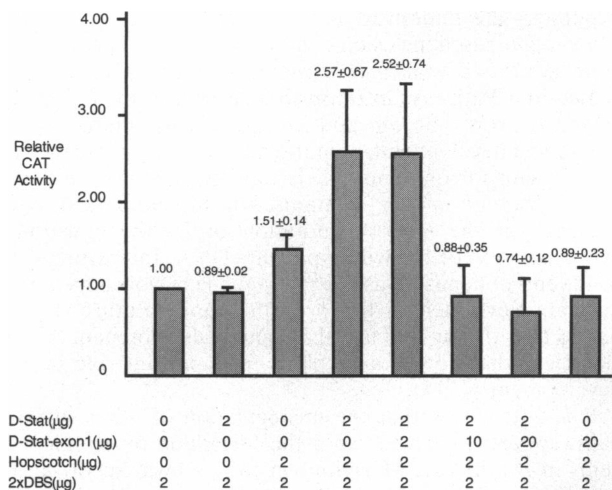

FIG. 4. Dominant negative roles of $stat^{HJ}$ DNA. The DNA used for transfection of Schneider cells is listed below the corresponding bar and the numbers given are the average of four experiments. The CAT activity obtained from the reporter construct, 2×DBS, is scored as 1.00.

product has some dominant negative effect on *hop*- and *D-Stat*-dependent transcriptional activation, including both transfected and putative endogenous STAT proteins. We have observed a similar dominant negative effect with a truncated mammalian Stat1 gene in U3A cell lines (R.Y. and J.E.D., unpublished data). Since the functional role of the N-terminal portion of Stat proteins is not delineated, it is not yet clear what the mechanism of such a dominant negative role might be.

DISCUSSION

The results reported here demonstrate that Jak kinase-Stat protein pathways are required for multiple processes during *Drosophila* development. Characterization of the $stat^{HJ}$ phenotype revealed that the Hop-D-Stat pathway regulates vein formation in the wing blade, an activity unexpected from previous analyses of *hop* mutants. Further, we observed other abnormalities, including segmentation defects and gaps in the dorsal tracheal trunks in $stat^{HJ}$ mutants. Finally, our recovery of the $stat^{HJ}$ and other mutations in an F_1 suppressor screen indcates that this genetic method should prove fruitful for identifying other components of *Drosophila* JAK–STAT signaling pathways.

The $stat^{HJ}$ Mutation. We provide several lines of evidence that $stat^{HJ}$ is indeed a mutation in the gene encoding the D-Stat protein. First, *D-Stat* and $stat^{HJ}$ map by *in situ* hybridization and by meiotic recombination to the same region of chromosome 3. Second, we find a point mutation in the *D-Stat* genomic sequence in the DNA from $stat^{HJ}$ that is not present in wild-type genomic DNA. The $stat^{HJ}$ point mutation results in two *D-Stat* mRNAs instead of one; translation of the long form would encode a truncated 41 amino acid protein that has a dominant negative effect in cell culture. In addition the total functional *D-Stat* mRNA is reduced about two-fold by the mutation. Third, overexpression of wild-type *D-Stat* by a heat shock promoter in transgenic flies can partially rescue the $stat^{HJ}$ phenotype.

The disruption of the splicing of the D-Stat transcript is apparently due to a C to T base change in the first intron within the protein coding region. While there is no exact precedent for mutations of the sort described here to give rise to aberrant splicing, experiments with animal viruses have shown that imperfect branch point mutations and mutations that lead to the introduction of SnRNP binding sites can lead to intron retention (18). The C to T mutation obvserved in the D-Stat intron could be such a mutation and experiments to test this hypothesis are underway. A similar differential splicing that leads to increased inclusion of a small coding segment of a *pax* gene occurs in a weak *aniridia* mutation found in humans (19).

Jak–Stat Pathways in *Drosophila* Development. We present clear evidence that *hop* and *stat* genetically interact during *Drosophila* development, as mutations in both loci can partially compensate for one another. Ectopic wing veins are consistently observed in $stat^{HJ}$ mutants, but are suppressed by the presence of the $hop^{Tum\text{-}l}$ mutation or by the constitutive overexpression of the wild-type *hop* cDNA. Interestingly, the wing veins of hemizygous $hop^{Tum\text{-}l}$; $stat^{+}$ survivors appear to be normal. Nevertheless, the $hop^{Tum\text{-}l}$ temperatuture-sensitive period lasts throughout larval and pupal development (5), and it is clear that *hop* protein plays an important role in wing development (4, 15).

It is noteworthy that the misregulation of other signaling pathways are known to cause the formation of ectopic wing veins in *Drosophila*. Mutations in the pathway involving the *Drosophila* epidermal growth factor receptor, Ras1, D-Raf, and the *rolled* MAP Kinase have been shown to cause ectopic posterior wing veins similar to those reported here for $stat^{HJ}$ (refs. 20 and 21, and references therein). In mammalian cells, a mitogen-activating protein kinase can modulate the activity of several STAT proteins by direct phosphorylation of the STAT proteins (22, 23). Hence, it is possible that there is also a direct interaction between the Hop-D-Stat and epidermal growth factor receptor pathways in the regulation of *Drosophila* wing veins, and possibly other patterning events. Ectopic wing veins are also seen in mutations involving the *Enhancer of split* pathway (24, 25). Experiments to examine the possible interactions of these pathways with that of Hop-D-Stat are currently underway.

An interaction between $hop^{Tum\text{-}l}$ and $stat^{HJ}$ is also evident in the higher survival rate of hemizygous $hop^{Tum\text{-}l}$ males. At present, the causes of $hop^{Tum\text{-}l}$ lethality at high temperatures are not certain. It is thought that hemocyte abnormalities contribute to the reduced viability (6, 26) although it is likely that abnormalities in other tissues, such as the gut, also play a major role. The results presented here indicate that hyperactivation of the Hop-D-Stat pathway contributes to $hop^{Tum\text{-}l}$ lethality. However, recent studies with a second dominant *hop* mutation suggest that the biochemical basis of the lethality may involve additional mechanisms (H.L. and C.R.D., unpublished observations).

Finally, *hop* and *D-Stat* interact to regulate the process of segmentation (7, 8), and the segmental abnormalities observed in $stat^{HJ}$ embryos are consistent with those in *hop* mutants. However, the strongest segmental defects seen in $stat^{HJ}$ embryos, including "pair-rule" cuticle patterns, have not been reported for null *hop* or *D-Stat* alleles. It is somewhat surprising that the $stat^{HJ}$ phenotype would be more extreme than that of *hop*, because it is likely that $stat^{HJ}$ mutants retain some level of STAT activity. It is possible that D-Stat activity is additionally required for signaling pathways distinct from JAK-STAT or Hop-D-Stat interactions. For example, D-Stat may be directly activated by receptor tyrosine kinases similarly to mammalian STATs (27), and/or Jak kinases other than Hop may exist in *Drosophila*. Alternatively, it is possible that the truncated $Stat^{HJ}$ protein can interfere with regulatory mechanisms in which the full-length Stat protein would normally not participate, consistent with the dominant negative effects of $stat^{HJ}$ overexpression observed in the tissue culture assays reported here.

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