Mutation in the Jak Kinase JH2 Domain Hyperactivates *Drosophila* and Mammalian Jak-Stat Pathways

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The Jak (Janus) family of nonreceptor tyrosine kinases plays a critical role in cytokine signal transduction pathways. In *Drosophila melanogaster*, the dominant *hop*^{Tum-1} mutation in the Hop Jak kinase causes leukemia-like and other developmental defects. Previous studies have suggested that the Hop^{Tum-1} protein might be a hyperactive kinase. Here, we report on the new dominant mutation *hop*^{T42}, which causes abnormalities that are similar to but more extreme than those caused by *hop*^{Tum-1}. We determined that Hop^{T42} contains a glutamic acid-to-lysine substitution at amino acid residue 695 (E695K). This residue occurs in the JH2 (kinase-like) domain and is conserved among all Jak family members. We determined that Hop^{Tum-1} and Hop^{T42} both hyperphosphorylated and hyperactivated D-Stat when overexpressed in *Drosophila* cells. Moreover, we found that the *hop*^{T42} phenotype was partially rescued by a reduction of wild-type *D-stat* activity. Finally, generation of the corresponding E695K mutation in murine Jak2 resulted in increased autophosphorylation and increased activation of Stat5 in COS cells. These results demonstrate that the mutant Hop proteins do indeed have increased tyrosine kinase activity, that the mutations hyperactivate the Hop–D-Stat pathway, and that *Drosophila* is a relevant system for the functional dissection of mammalian Jak-Stat pathways. Finally, we propose a model for the role of the Hop–D-Stat pathway in *Drosophila* hematopoiesis.

A major advance in understanding cytokine signal transduction pathways has come from recent studies on the Jak (Janus) family of nonreceptor tyrosine kinases (reviewed in references 18, 19, and 36). These proteins are structurally different from other cytoplasmic protein tyrosine kinases, as they contain a kinase-like (JH2) domain adjacent to the carboxy-terminal kinase (JH1) domain and lack src homology 2 (SH2) and SH3 domains. Jak kinases associate with the intracellular domains of a large number of cytokine and growth factor receptors, and their roles in intracellular signaling can generally be explained as follows. After the extracellular ligand binds to its receptor, the Jak kinases become rapidly phosphorylated on tyrosine. They then phosphorylate the receptor, which provides docking sites for latent cytoplasmic transcription factors termed Stat (for signal transducers and activators of transcription) proteins. The Jak kinases then phosphorylate the Stat proteins, which in turn dissociate from the receptor complex, form homodimers or heterodimers, and translocate to the nucleus. There, the Stat proteins participate in the transcriptional activation of ligand-inducible genes. The Jak-Stat pathway was first demonstrated for interferon signaling pathways (7, 42) and was soon after found to participate in other cytokine and growth factor pathways (2, 18, 46). Four distinct Jak kinases and six Stat proteins have been identified in mammals (18, 19).

Mammalian tissue culture cells have been successfully used to identify Jak-Stat interactions and activation. However, recent studies using transgenic mice have demonstrated the importance of in vivo model systems for the examination of Jak

kinases and Jak-Stat pathways. For example, an examination of Stat1-deficient mice demonstrated that the role of Stat1 is apparently restricted to innate immunity (9, 28). This result was unexpected, because Stat1 is activated in response to several cytokines in tissue culture cells. Furthermore, Stat6-deficient mice display abnormal interleukin-4 (IL-4) signaling, resulting in defective Th2 helper T-cell differentiation (20, 38, 40). IL-3 also activates Stat6 in various tissue culture cell lines, but Stat6-deficient mice displayed no abnormalities in myeloid proliferation or signaling. Disruption of the Jak3 gene, which is expressed primarily in hematopoietic cells, resulted in defective lymphoid signaling in transgenic mice (32, 41).

The fruit fly, *Drosophila melanogaster*, provides an excellent genetic system for studying Jak kinases and Jak-Stat pathways in vivo. Indeed, both components of the pathway have been identified in *Drosophila*. A fruit fly Jak kinase is encoded by the *hopscotch* (*hop*) locus (5). Mutants lacking *hop* activity have a number of abnormalities, including the loss of segmental structures and a failure of diploid imaginal precursor cells to proliferate (33). Further, a *Drosophila stat* gene, *D-stat*, has recently been characterized (16, 47, 48). The loss or reduction of *D-stat* activity also leads to segmental defects as well as to the formation of ectopic wing veins. In the embryo, D-Stat participates in the transcriptional activation of the *even-skipped* segmentation gene.

Hop is also involved in hematopoiesis in *Drosophila*. *hop*^{Tum-1} is a dominant, temperature-sensitive mutation that causes leukemia-like defects (12). In *hop*^{Tum-1} mutants, there is an overproliferation of phagocytic larval plasmatocytes at all culture temperatures. At high culture temperatures, these hemocytes also prematurely differentiate into lamellocytes, aggregate, and form melanotic masses. In addition, the *hop*^{Tum-1} mutation causes melanotic-tumor formation and other abnormalities in nonhematopoietic tissues (12). Hop^{Tum-1} contains

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an amino acid change of glycine to glutamic acid at residue 341, located near the amino-terminal end of the Hop JH4 domain (26). This residue is not well conserved among Jak family members.

The phenotype of the *hop*^{Tum-1} mutation suggested that the Hop^{Tum-1} protein might be hyperactive (11). This hypothesis was supported by experiments in which Hop^{Tum-1}, when overexpressed in Schneider cells, was found to be hyperphosphorylated on tyrosine compared to wild-type Hop (14). However, it was formally possible that hyperphosphorylation of Hop^{Tum-1} might not correlate with increased kinase activity. Therefore, it remained to be proven that Hop^{Tum-1} could hyperactivate an appropriate substrate and to determine the identity of that substrate.

In the work described in this paper, we attempted to address these questions and to better understand the roles of *hop* in hematopoiesis. We first conducted a genetic screen for additional dominant *hop* mutations and identified the *hop*^{T42} mutation. *hop*^{T42} causes abnormalities that are similar to but more extreme than those caused by *hop*^{Tum-1}. We determined that Hop^{T42} contains a glutamic acid-to-lysine substitution at amino acid residue 695 (E695K). This residue occurs in the JH2 (kinase-like) domain and is conserved among all known Jak kinases. We determined that Hop^{Tum-1} and Hop^{T42} both hyperphosphorylated and hyperactivated D-Stat when overexpressed in tissue culture cells. Moreover, we found that the lethality and melanotic-tumor formation in *hop*^{T42} mutants were partially rescued by *D-stat* loss-of-function mutations. Finally, generation of the corresponding E695K mutation in murine Jak2 caused it to hyperactivate Stat5 in COS cells. Therefore, we have verified that the dominant *Drosophila hop* mutations do indeed cause Hop to be a hyperactive kinase and that they cause hyperactivation of the Hop-D-Stat pathway. We propose that dominant Jak kinase mutations in mammals could contribute to the potential for leukemia in vivo.

MATERIALS AND METHODS

Fly culture and phenotypic analyses. Flies were grown on standard cornmeal-molasses-agar-yeast medium supplemented with Tegosept and live yeast. Mutations include *yellow* (*y*; 1-0.0), *vermillion* (*v*; 1-33.0), *hop* (1-34.5), *miniature* (*m*; 1-36.1), *D-stat* (3-68.9), and the Basc balancer chromosome. The wild-type strain used was Oregon R. The mutations are described in references 16, 25, and 48. Blood cell counts were performed as described in reference 50. Histological sections were obtained as described in reference 8 and were stained with Feulgen and fast green (17).

Isolation of the dominant *hop*^{T42} mutant. A *y* stock was made isogenic for the X chromosome. *y* males were mutagenized with ethyl methanesulfonate by the adult-feeding method (24) and mated to Basc females. The potentially mutagenized *y* (*hop*^{*})/Basc females were mated individually to Basc males at 29°C. The progeny were examined for the presence of melanotic masses in the heterozygous *y* (*hop*^{*})/Basc females and for the presence or absence of *y* (*hop*^{*}) males. The meiotic-map position of recovered mutations was determined by recombination mapping with the *v* and *m* markers. One mutation that maps to the *hop* locus, labelled *T42*, was recovered.

DNA sequence analyses and cloning of *hop*^{T42}. RNA isolation, reverse transcription-PCR, and direct sequencing of the PCR products were described previously (26). We sequenced both *hop*^{T42} and its parental isogenic *y* stock DNA. To construct *hop*^{T42} cDNA, the wild-type *hop* cDNA in pBluescript SK (+) was digested by *Eco*RI, dephosphorylated, and ligated with the *Eco*RI fragment amplified from *hop*^{T42} mRNA. The construct was sequenced to verify potential mutations created by PCR. To prove that the E695K substitution in Hop is responsible for the phenotype of *hop*^{T42}, we performed transgenic-fly experiments. However, we were unable to obtain stable transgenic-fly stocks containing an actin 5C promoter-*hop*^{T42} cDNA construct. These results were similar to our previous results with an actin 5C-*hop*^{Tum-1} construct (26).

DNA constructs. To express Hop in the baculovirus system, *hop* cDNAs were subcloned into the transfer vector pAcSG2 (PharMingen) by *Xho*I and *Not*I digestions. For transfections in Schneider L2 cells, *hop* cDNAs were subcloned into pPac5C by *Kpn*I and *Not*I digestions. pPac5C-*D-stat* cDNA, containing a Flag epitope tag at the carboxy terminus, was the kind gift of Riqiang Yan and James Darnell (47). The kinase-like domain deletion mutant *hop* (Δ JH2) was constructed in two steps. First, a *Pst*I digestion of the wild-type *hop* cDNA in

pBluescript SK (+) removed the entire kinase-like domain and a portion of the kinase domain. Second, a PCR product containing the deleted portion of the kinase domain was digested with *Pst*I and ligated with the *Pst*I-digested *hop* cDNA. The construct was sequenced and had a deletion from arginine 567 to methionine 878.

pBOS-Jak2 Δ VIII and pBOS-Jak2 were kindly provided by Don Wojchowski (49). Two hemagglutinin epitopes were inserted at the amino terminus of Jak2 by PCR. The mutant Jak2 clone was generated by PCR mutagenesis, and the mutation was confirmed by sequence analysis. pXM-ovine Stat5 cDNA was a kind gift of Bernd Groner (44). Murine Stat5a cDNA (3a), the kind gift of Jim Ihle, was subcloned into the vector pcDNA3 for expression in COS cells.

Antisera. Anti-Hop polyclonal antibodies were raised in rabbits against the carboxy-terminal sequence EVATKVTHPTDG, which was coupled through the terminal cysteine thiol to keyhole limpet hemocyanin. Crude anti-Hop antibodies were purified through an affinity purification column containing the peptide antigen. Antiphosphotyrosine monoclonal antibody 4G10 and anti-Jak2 polyclonal antisera were purchased from Upstate Biotechnology, Inc. Anti-phosphorylated-Stat5 antibody was the kind gift of David Frank. Peptide-specific anti-Stat5 monoclonal antibodies were kindly provided by Markus Heim, and anti-Stat5 monoclonal antibody was also purchased from Transduction Laboratories. Anti-Flag antibody M2Ab and anti-Flag M2 affinity gels were obtained from IBI, Kodak.

Baculovirus expression of Hop proteins in Sf21 insect cells. *hop* cDNAs were subcloned into the baculovirus transfer vector pAcSG2. In vivo recombination with Bac PAK6 viral genomic DNA (Clontech) yielded virus stocks which were highly enriched for recombinant virus. The recombinant virus was plaque purified and amplified twice to a high-titer stock by standard methods (39). Infected Sf21 cells were lysed in lysis buffer (50 mM Tris [pH 8.0], 137 mM NaCl, 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 30 μ g of aprotinin, and 1 mM Na₃VO₄). Fifty micrograms of protein lysates was separated by sodium dodecyl sulfate-8% polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to polyvinylidene difluoride membranes. The blots were probed with anti-Hop antibody, stripped, and reprobed with antibody 4G10. An enhanced chemiluminescence kit (Amersham) was used to record the signals.

Schneider cell transfections. Schneider L2 cells were cultured at 25°C in 1× *Drosophila* medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. The transfection experiments were performed by the calcium precipitation procedure (22) with the following modifications. Cells were seeded on 100-mm-diameter dishes at a density of 2×10^5 to 3×10^5 cells per ml in a total volume of 7 ml. Cells were transfected with 4 μ g of wild-type *hop* cDNA or 6 μ g of mutant *hop* cDNAs along with 14 μ g of *D-stat* cDNA. The transfection was done in duplicate plates. Forty-eight hours after transfection, the cells were washed once in phosphate-buffered saline (PBS) plus 2 mM Na₃VO₄. Cells were lysed in a solution containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1.0% Triton X-100, 10 mM Na₂P₂O₇, 10 mM NaF, 1 mM Na₃VO₄, 5 mM EDTA, 1 mM PMSF, 5 μ g of leupeptin per ml, 5 μ g of pepstatin A per ml, and 5 μ g of aprotinin per ml. Lysates were incubated on ice for 5 min and then cleared by centrifugation (4). We used 100 μ g of proteins for anti-Hop Western blot analyses. For immunoprecipitation experiments, D-Stat was precipitated for 2 h from 600 μ g of protein lysates by using anti-Flag M2 affinity gels. The immunoprecipitates were washed three times with cold PBS and boiled in 1× sample buffer before being loaded for SDS-PAGE. The blot was first probed with antibody 4G10 and, after being stripped, was reprobed with anti-Flag antibody M2Ab.

COS cell transfections. COS-7 cells were transfected by the DEAE-dextran-chloroquine method (37). Briefly, cells were incubated for 3.5 h in DEAE-dextran-chloroquine with various amounts of DNA as indicated in the figure legends. Cells were then incubated in PBS-10% dimethyl sulfoxide for 2 min, washed once in PBS, and incubated in Dulbecco modified Eagle medium for 24 h before harvest. The plates were washed once in a solution containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM Na₂P₂O₇, 10 mM NaF, 1 mM Na₃VO₄, and 5 mM EDTA. Lysates were obtained as described above (4). We used 100 μ g of protein lysates for Western blot analyses. The blot was first probed with antibody 4G10 and, after being stripped, was sequentially reprobed with anti-Jak2 and anti-Stat5 antibodies. In separate experiments, Jak2 was immunoprecipitated with anti-Jak2 antibodies (Upstate Biotechnology, Inc.) and subsequently detected by 4G10 and anti-Jak2 antibodies. In addition, an antibody specific to tyrosine-phosphorylated Stat5 (4a) was used in Western blots to detect tyrosine phosphorylation of murine Stat5a.

Electrophoretic mobility shift assays (EMSA). Nuclear extracts were prepared from cotransfected Schneider cells as described previously (23). For COS-7 cell transfections, nuclear extracts were prepared as described in reference 1. Briefly, cell pellets were resuspended in 1.0 ml of buffer A (10 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol, 1 mM PMSF, 5 μ g of leupeptin per ml, 5 μ g of pepstatin A per ml, and 5 μ g of aprotinin per ml) and allowed to swell on ice for 10 min. Cells were then vortexed for 10 s and centrifuged for 10 s, and the supernatant was discarded. The pellet was resuspended in buffer A, vortexed, and centrifuged, and the supernatant was discarded. Nuclear proteins were resuspended in 100 μ l of a solution containing 20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM PMSF, 5 μ g of leupeptin per ml, 5 μ g of pepstatin A per ml, and 5 μ g of aprotinin per ml.

TABLE 1. Survival and tumor formation in *hop* mutants

Cross	Temp (°C)	Total no. of adults	No. (%) of expected <i>hop</i> ; (+ or <i>D-stat</i> ⁶³⁴⁶)/+ males ^a	<i>hop</i> /+; (+ or <i>D-stat</i> ⁶³⁴⁶)/+ females			
				No.	% with no tumors	% with small tumors	% with large tumors
<i>y cv v hop</i> ^{T42} /Basc × Oregon R	29	2,479	0 (0.0)	711	2.8	12.4	84.8
<i>y cv v hop</i> ^{T42} /Basc × <i>D-stat</i> ⁶³⁴⁶ /TM3	29	2,717	0 (0.0)	637	47.4	47.6	5.0
<i>y v hop</i> ^{Tum-1} /Basc × Oregon R	29	2,586	42 (6.0)	940	21.3	53.2	25.5
<i>y v hop</i> ^{Tum-1} /Basc × <i>D-stat</i> ⁶³⁴⁶ /TM3	29	3,640	174 (31.9)	772	62.8	35.1	2.1
<i>y cv v hop</i> ^{T42} /Basc × Oregon R	25	2,445	13 (1.9)	893	61.3	35.7	3.0
<i>y cv v hop</i> ^{T42} /Basc × <i>D-stat</i> ⁶³⁴⁶ /TM3	25	2,159	53 (15.7)	414	95.7	4.3	0.0
<i>y v hop</i> ^{Tum-1} /Basc × Oregon R	25	2,613	248 (42.8)	926	93.2	6.7	0.1
<i>y v hop</i> ^{Tum-1} /Basc × <i>D-stat</i> ⁶³⁴⁶ /TM3	25	3,257	511 (>100)	550	93.1	6.5	0.4
<i>y cv v hop</i> ^{T42} /Basc × Oregon R	17	2,343	334 (51.6)	ND ^b			
<i>y cv v hop</i> ^{Tum-1} /Basc × Oregon R	17	2,041	579 (>100)	ND			

^a Calculated relative to the number of Basc male siblings.^b ND, not determined.

DNA binding experiments were performed as described previously (35) with a double-stranded ³²P-labelled oligonucleotide specific for either D-Stat binding (47) or Stat5 binding (AGATTCTAGGAATTCAAATC, which was derived from the β -casein promoter). Two micrograms of nuclear extract was mixed with 0.25 ng of the ³²P-labelled oligonucleotide in 20 μ l of binding buffer [13 mM HEPES (pH 7.9), 65 mM NaCl, 1 mM dithiothreitol, 0.15 mM EDTA, 8% glycerol, 50 μ g of poly(dI-dC) per ml] for 20 min at 4°C. For antibody binding experiments, M2Ab or a peptide-specific Stat5 antibody was incubated at the end of the binding reaction for an additional 20 min at 4°C. For competition experiments, a 50-fold excess of the unlabelled specific oligonucleotide was included in the binding reaction. DNA-protein complexes were separated on a 5% polyacrylamide gel in 0.5× Tris-borate-EDTA and visualized by autoradiography.

RESULTS

Isolation and characterization of the dominant *hop* mutation, *hop*^{T42}. We conducted a chemical mutagenesis screen to isolate additional dominant mutations in the *hop* locus (see Materials and Methods). One new allele, *hop*^{T42}, was recovered. The phenotype of *hop*^{T42} is similar to that of the dominant *hop*^{Tum-1} mutation (12). Both *hop*^{T42} and *hop*^{Tum-1} are temperature sensitive, with more severe defects observed at higher culture temperatures (Table 1). The survival of *hop*^{T42} hemizygotes is reduced at all culture temperatures tested. We recovered 51.6% of the expected number of hemizygous *hop*^{T42} males at 17°C, 1.9% at 25°C, and 0% at 29°C (Table 1). In contrast, we recovered hemizygous *hop*^{Tum-1} males at a higher rate: >100% at 17°C, 42.8% at 25°C, and 6.0% at 29°C (Table 1). *hop*^{T42} mutants also contain melanotic masses (Fig. 1), and the tumor sizes are also larger than in *hop*^{Tum-1} mutants at both culture temperatures examined (Table 1). The extent of *hop*^{T42} melanotic-tumor formation varies among individuals, and melanotic tumors can result from hematopoietic abnormalities (Fig. 1A), from the melanization of gastric ceca and gut tissue (Fig. 1C), or from both types of abnormalities (Fig. 1B).

We also examined tissue sections of *hop*^{T42} hemizygous male larvae. We observed that the gastric ceca and gut tissue were frequently overgrown, misshapen, and/or partially melanized (Fig. 2B). Some individuals had regions of missing tissue in the brain lobes (Fig. 2C), misshapen anterior spiracles, and/or reductions in the amount of fat body tissue. It is likely that these nonhematopoietic abnormalities, as well as the hematopoietic cell defects, contribute to the lethality observed in the mutants.

In the hematopoietic system, the *hop*^{T42} mutation causes leukemia-like defects (Fig. 3 and Table 2). At all culture temperatures, we observed an overproliferation of phagocytic plasmatocytes in *hop*^{T42} mutant larvae (Fig. 3B and C). At high

culture temperatures, many plasmatocytes also prematurely differentiated into lamellocytes and aggregated into masses (Fig. 3C). These masses can include hundreds of hemocytes and often become melanized.

***hop*^{T42} has an E695K amino acid substitution in the JH2 domain.** To characterize the molecular lesion in *Hop*^{T42}, we isolated poly(A)⁺ RNA from third-instar larvae of hemizygous *hop*^{T42} males and performed reverse transcription-PCR to amplify the entire protein-coding region. The amplified PCR products were purified by agarose gel electrophoresis and sequenced directly. DNA from the *y* parental stock used in the *hop*^{T42} mutagenesis screen was also sequenced. Sequence analysis of the cDNAs indicated that *Hop*^{T42} contains a single amino acid change of glutamic acid to lysine at residue 695. The *y* parental stock, the *hop*^{Tum-1} stock, and other wild-type *hop* cDNAs all encode glutamic acid at this position (5, 26). The E695K mutation occurred in the JH2 (kinase-like) domain of the Jak kinase (Fig. 4A). Sequence alignment of *Hop*^{T42} with other Jak kinases indicated that the E695 residue in *Hop* is conserved among all known Jak kinase family members (Fig. 4B).

FIG. 1. Melanotic-tumor phenotypes of *hop*^{T42} mutants. Three examples of *hop*^{T42} hemizygous male larvae, cultured at 29°C, are shown. (A) Larva with numerous small melanotic masses consisting of aggregated hemocytes (plasmatocytes and lamellocytes). Most of these masses were observed to circulate throughout the hemolymph. (B) Larva with tumors of the gut and gastric ceca in addition to masses of melanized hemocytes. This combination of melanotic tumors represents the phenotype most commonly observed in the mutant larvae. (C) Larva with a large melanized tumor consisting primarily of gut tissue.

FIG. 2. Melanotic tumors in sections of *hop*^{T42} mutant larvae. Individuals were cultured at 29°C, embedded in Paraplast, sectioned, and stained with Feulgen and fast green. (A) Wild-type Oregon R larva. Note the characteristic shapes and locations of the proventriculus, gastric ceca, and anterior midgut tissues. (B) *hop*^{T42} mutant larva. The gastric ceca and anterior midgut form a large, partially melanized tumor surrounding the proventriculus and esophagus. Fat body tissue is present in a smaller amount, and has less regular borders, than in wild-type larvae. (C) *hop*^{T42} mutant larva. Note the vacuole in the brain lobe. The anteriors of the larvae are at the top. (D through F) B, brain lobe; C, gastric ceca; E, esophagus; F, fat body; G, gut tissue; GT, tumor of the gut and/or gastric ceca; I, imaginal disc; P, proventriculus.

Both Hop^{Tum-1} and Hop^{T42} hyperactivate the *Drosophila* Stat protein. Hop^{Tum-1} has been predicted to be a hyperactive kinase because of genetic data (11, 26) and because the protein was itself hyperphosphorylated on tyrosine when overexpressed in *Drosophila* Schneider cells (14). Based on the phenotypic analysis described above, we predicted that Hop^{T42}

would be similarly hyperactive. We tested these predictions for both Hop^{Tum-1} and Hop^{T42}. First, the wild-type and mutant *hop* cDNAs were subcloned into the baculovirus transfer vector pAcSG2 and the proteins were overexpressed in *Sf21* insect cells. Total lysates of infected cells were separated by SDS-PAGE and then analyzed by Western blotting. The blots were

FIG. 3. Leukemia-like abnormalities in *hop*^{T42} mutants. (A) Circulating blood cells obtained from a wild-type Oregon R larva. Several plasmatocytes are seen. (B) Blood cells from a *hop*^{T42} larva, cultured at 17°C. There is a significant increase in the number of hemocytes, although the differentiation and aggregation of these plasmatocytes are limited. (C) Part of an aggregated mass of blood cells obtained from a *hop*^{T42} larva cultured at 29°C. The aggregation consists primarily of differentiated lamellocytes but also includes plasmatocytes.

TABLE 2. Circulating-hemocyte concentrations

Strain	Temp (°C)	No. of individuals	Total hemocytes (10 ⁶)/ml ^a	% Lamellocytes
Oregon R	17	10	0.69 ± 0.18	6.0
<i>hop</i> ^{Tum-1} mutant	17	10	34.29 ± 4.96 ^b	5.7
<i>hop</i> ^{T42} mutant	17	10	24.64 ± 5.09 ^b	8.6
Oregon R	29	10	0.37 ± 0.05	3.1
<i>hop</i> ^{Tum-1} mutant	29	9	8.31 ± 0.94 ^b	62.4 ^b
<i>hop</i> ^{T42} mutant	29	10	8.45 ± 0.48 ^b	72.7 ^b

^a Means ± standard errors.

^b Significantly different from Oregon R control (*P* < 0.01).

first probed with anti-Hop polyclonal antisera to normalize the expression levels (Fig. 5A). The same blots were then stripped and reprobed with antiphosphotyrosine monoclonal antibody 4G10 (Fig. 5A). The results indicated that Hop^{T42} was hyperphosphorylated on tyrosine and further confirm the findings (14) that Hop^{Tum-1} was similarly hyperphosphorylated.

We then directly tested whether Hop^{Tum-1} and Hop^{T42} can hyperphosphorylate and hyperactivate the *Drosophila* Stat protein (Fig. 5). Both *hop* and *D-stat* cDNAs were subcloned into the vector pPac5C and transiently coexpressed in *Drosophila* Schneider cells. A Flag epitope tag was included at the C-terminal end of D-Stat to facilitate detection and purification of D-Stat (47). After cotransfection, D-Stat was immunoprecipitated from total lysates with anti-Flag antibody M2Ab and examined by Western blot analyses (Fig. 5B). In addition, a portion of the total lysates was separately examined on Western blots with anti-Hop polyclonal antisera (Fig. 5C). The data indicated that the two mutant Hop kinases hyperphosphorylated D-Stat on tyrosine, in comparison to the wild-type Hop control, even though more wild-type Hop protein was observed.

We next tested whether hyperphosphorylation of D-Stat correlated with an increased DNA binding activity of D-Stat, using EMSA (Fig. 5D). Nuclear proteins were isolated from the transfected cells and incubated with ³²P-labelled oligonu-

cleotides containing a consensus D-Stat binding site (47), in the presence of anti-Flag antibody M2Ab. The coexpression of the mutant Hop proteins caused a significant increase in the DNA binding activity of D-Stat. Two supershifted bands, A and B, were observed. These two bands probably represent the Flag-tagged D-Stat homodimer (A) and a heterodimer with an endogenous Stat protein (B), as proposed previously (47). The appearance of supershifted bands was dependent upon exogenous Hop activity and was abolished by competition with unlabelled D-Stat binding sequences. In the absence of the anti-Flag antisera, we also observed two weaker gel shift bands whose intensities were increased in cells cotransfected with a mutant rather than a wild-type *hop* expression vector (data not shown). Therefore, our results demonstrated that both Hop^{Tum-1} and Hop^{T42} hyperactivated D-Stat.

To further explore the role of the JH2 domain, we tested the kinase activity of a Hop protein lacking the entire JH2 region (ΔJH2) (Fig. 5B to D). Deletion of the JH2 domain caused a drastic reduction of tyrosine phosphorylation and activation of D-Stat, even though Hop(ΔJH2) accumulated to levels comparable to those of Hop^{Tum-1} and Hop^{T42}. In addition, Hop (ΔJH2) itself was not noticeably tyrosine phosphorylated, as determined by Western analysis of total cell lysates (data not shown). Similarly, deletion of the JH2 domain from Tyk2 also abolished its catalytic activity (43). Thus, our results indicate that the JH2 domain is required for activation of Hop and that the E695K mutation does not simply eliminate the function of the Hop JH2 domain.

A point mutation corresponding to Hop^{T42} hyperactivates murine Jak2. Since the E695K substitution in Hop^{T42} occurred at a highly conserved amino acid residue (Fig. 4B), we could test whether the corresponding mutation would similarly hyperactivate the kinase activity of a mammalian Jak kinase. We generated such a mutation in murine Jak2(E665K) and tested the effects of the mutant Jak2 protein on autophosphorylation and Stat5 activation by COS cell cotransfection assays (Fig. 6). Total lysates of transfected cells were used for Western blot analyses (Fig. 6A). The blots were first probed with antibody 4G10. Both the wild-type and the mutant Jak2 proteins showed significant autophosphorylation and phosphorylation of ovine

FIG. 4. (A) Schematic structure of Hop, showing the location of the dominant Hop^{Tum-1} and Hop^{T42} amino acid substitutions. The conserved Jak kinase domains are named according to the system of Harpur et al. (13). (B) Sequence alignment within the JH2 domain among the Jak family members. The glutamic acid mutated in the Hop^{T42} protein (boxed) is highly conserved among known Jak kinases. Residues identical to those in Hop are shown in bold.

FIG. 5. Hop^{Tum-1} and Hop^{T42} hyperactive D-Stat in tissue culture cells. (A) Hop^{Tum-1} and Hop^{T42} are hyperphosphorylated on tyrosine when overexpressed in *Sf21* insect cells. Shown are immunoblots of total cellular lysates of overexpressed wild-type Hop (Wt), Hop^{Tum-1} (Tum-1), and Hop^{T42} (T42) as well as nonrecombinant lysate (-). The blot was first probed with anti-Hop antisera (bottom), then stripped, and reprobed with antiphosphotyrosine antibody 4G10 (top). (B) Hop^{Tum-1} and Hop^{T42} hyperphosphorylate D-Stat in Schneider cells. D-Stat and Hop were overexpressed as indicated. D-Stat was immunoprecipitated from total lysates with anti-Flag epitope antibody (M2Ab). The immunoprecipitates were analyzed by immunoblotting sequentially with 4G10 (top) and M2Ab (bottom). ΔJH2 lacks the JH2 domain of Hop. IgG, immunoglobulin G. (C) Control for Hop protein overexpression. A portion of the total lysates obtained for the experiments in panel B was used for anti-Hop Western blot analysis. (D) Hop^{Tum-1} and Hop^{T42} hyperactivate the DNA binding activity of D-Stat. The binding of D-Stat to its consensus DNA recognition element was tested in the presence of anti-Flag epitope antibody (M2Ab) by EMSA. Two supershifted bands were seen, possibly corresponding to the homodimer (arrow A) and heterodimer (arrow B) as proposed previously (47). In lanes 7 to 9, excess amounts of unlabelled D-Stat binding oligonucleotides were included as specific competitors (sp). Ab, antibody.

Stat5. However, the mutant Jak2 protein showed significantly higher levels of tyrosine autophosphorylation as well as moderately higher levels of Stat5 phosphorylation (Fig. 6A; compare lanes 1 and 2 and lanes 3 and 4). As a control, the expression of a kinase-inactive form of Jak2 (DI, for dominant-inhibitory) yielded no detectable tyrosine phosphorylation of the Jak kinase and showed a reduction of the phosphorylation of Stat5 (Fig. 6A, lane 5) compared with Stat5 transfection alone (Fig. 6A, lane 6), presumably due to the dominant negative effect of this Jak kinase (49). The blots were also stripped and reprobed sequentially with anti-Jak2 and anti-Stat5 antibodies. Similar expression levels were observed for each Jak2 and Stat5 cotransfection (Fig. 6A, bottom).

To further confirm that the Jak2(E665K) protein is hyper-

active, we performed additional experiments. Overexpressed Jak2 proteins were immunoprecipitated from total cell lysates and detected sequentially with 4G10 and anti-Jak2 antibodies (Fig. 6B). Again, mutant Jak2 had increased tyrosine phosphorylation (Fig. 6B, lanes 2 and 4) compared to wild-type Jak2 (Fig. 6B, lanes 1 and 3). Next, we utilized an activation-specific anti-Stat5 antibody that specifically recognizes the phosphorylated form of Stat5 (4a) to examine the effects of Jak2(E665K) on overexpressed murine Stat5a (Fig. 6C). Consistent with the previous results, overexpressed mutant Jak2 led to an increase in Stat5a tyrosine phosphorylation (Fig. 6C, lanes 1, 3, and 5) compared to wild-type Jak2 (Fig. 6C, lanes 2, 4, and 6). Together, the data indicate that the glutamic acid-to-lysine mutation in murine Jak2 which corresponds to that in

FIG. 6. Mutant murine Jak2 hyperactivates Stat5 in COS-7 cells. (A) Mutant Jak2(E665K) has enhanced tyrosine phosphorylation activity. Cells were cotransfected with ovine Stat5 and wild-type Jak2 (wt), mutant Jak2(E665K) (mt), or a kinase-inactive Jak2 (DI). Total lysates were analyzed by immunoblotting with antiphosphotyrosine antibody 4G10 (pTyr blot). The blot was stripped and reprobed with a peptide-specific Jak2 antibody (Jak2 blot) and then with a peptide-specific Stat5 antibody (Stat5 blot). We added 0.5 μ g (lanes 1 and 2) or 0.75 μ g (lanes 3 and 4) of Jak2 cDNA in each transfection, and 5 μ g of Stat5 cDNA was also included in each transfection. Note that in the top panel Jak2 migrates slightly further than an unknown protein whose expression is not affected. (B) Examination of immunoprecipitated Jak2 proteins. Wild-type and mutant Jak2 were overexpressed, immunoprecipitated with anti-Jak2 antibodies, and immunoblotted. We added 0.5 μ g (lanes 1 and 2) or 0.75 μ g (lanes 3 and 4) of Jak2 cDNA and 1.0 μ g of Jak2 (DI) cDNA (lane 5). (C) Jak2(E665K) hyperphosphorylates murine Stat5a. Cells were transfected with 0.5 μ g (lanes 1, 2, 7, and 8), 0.75 μ g (lanes 3 and 4), or 1.00 μ g (lanes 5, 6, 9, and 10) of Jak2 cDNA; 0.5 μ g (lanes 7 and 8) or 1.0 μ g (lanes 9 and 10) of Jak2 (DI) cDNA; and 2.5 μ g of Stat5 cDNA (all lanes except lane 11). (D) Stat5 activation is increased by the mutant Jak2(E665K). The shifted band (lower arrow) and supershifted band (upper arrow) are indicated. Nuclear extracts were prepared from COS-7 cells and analyzed by EMSA. Gel shift complexes were detected by binding to a 32 P-labelled β -casein oligonucleotide. Reactions were analyzed on a 5% acrylamide gel and detected by autoradiography. Concentrations of transfected DNA are the same as in panel A for lanes 1 to 7, with nuclear proteins from the 0.5- μ g Jak2 DNA transfections used for the competition and supershift experiments shown in lanes 8 to 13. sp, addition of cold β -casein oligonucleotides; proteins, ns, addition of nonspecific competitor oligonucleotides. Ab, antibody.

Drosophila Hop^{T42} generates an enzyme with increased tyrosine kinase activity.

We next used EMSA to correlate Stat5 phosphorylation levels with DNA binding activity (Fig. 6D). Compared to coexpression of wild-type Jak2 and Stat5 (Fig. 6D, lanes 1 and 3), coexpression of the mutant Jak2 and Stat5 led to an increased amount of Stat5 bound to a DNA probe from the β -casein promoter (Fig. 6D, lanes 2 and 4). The gel shift band was specific to Stat5 complexes, as it was competed by an unlabelled β -casein probe (Fig. 6D, lanes 8 and 10) but not by a

nonspecific oligonucleotide (Fig. 6D, lanes 9 and 11). In addition, the complex was supershifted by Stat5-specific antibodies (Fig. 6D, lanes 12 and 13), whereas antibodies to other Stat proteins failed to supershift this complex (data not shown). In control experiments, no Stat5 binding activity was seen in cells expressing the kinase-inactive Jak2 (DI) and Stat5 (Fig. 6D, lane 5), Stat5 alone (Fig. 6D, lane 6), or mock-transfected cells (Fig. 6D, lane 7). Thus, we have demonstrated that the mutant murine Jak2 protein is also a hyperactive kinase that hyperactivates the DNA binding activity of Stat5.

A reduction of D-Stat activity in vivo suppresses the lethality and melanotic-tumor formation associated with *hop*^{T42}. The biochemical data described above demonstrate that both Hop^{Tum-l} and Hop^{T42} can hyperphosphorylate and hyperactivate D-Stat in Schneider cells. We took a genetic approach to determine the in vivo relevance of this interaction for the *hop*^{T42} mutation. Two *D-stat* mutations have been identified: the strong loss-of-function allele, *D-stat*⁶³⁴⁶ (16), and the hypomorphic, partially dominant negative allele, *D-stat*^{HJ} (47, 48). *D-stat*⁶³⁴⁶ mutants die at the end of embryogenesis or as young larvae, whereas *D-stat*^{HJ} mutants can survive to adulthood. Both mutations cause embryonic segmentation defects similar to those seen in loss-of-function *hop* mutations (16, 48). Additional defects can be readily observed in *D-stat*^{HJ} mutant adults, including adult segmentation abnormalities and the formation of ectopic wing veins (48).

We tested whether the dominant phenotype associated with *hop*^{T42} would be suppressed by a reduction in *D-stat* activity. We found that one copy of *D-stat*⁶³⁴⁶ partially suppressed the phenotype of *hop*^{T42} (Table 1). At 25°C, both the mortality of *hop*^{T42} hemizygous males and tumor formation in heterozygous females were notably reduced by the *D-stat*⁶³⁴⁶ mutation. At 29°C, melanotic-mass formation in *hop*^{T42} heterozygous females was also reduced by *D-stat*⁶³⁴⁶. However, the effects of the *hop*^{T42} mutation are sufficiently severe at this temperature that male lethality is not rescued by *D-stat*⁶³⁴⁶. The formation of melanotic masses and the reduced viability of *hop*^{Tum-l} and *hop*^{T42} mutants correlate with the premature differentiation and aggregation of hemocytes. The *D-stat*⁶³⁴⁶ and *D-stat*^{HJ} mutations can suppress the premature differentiation and aggregation, but not the overproliferation, of *hop*^{Tum-l} and *hop*^{T42} larval hemocytes (26a).

The suppression of the *hop*^{T42} phenotype by *D-stat*⁶³⁴⁶ was less than its suppression of the *hop*^{Tum-l} phenotype (Table 1). This result is consistent with the more severe defects caused by *hop*^{T42} in a background that is wild type for *D-stat* (Table 1). Our data also support previous studies that indicated a genetic interaction between *hop*^{Tum-l} and *D-stat* mutant alleles. In particular, it was shown that *hop*^{Tum-l} can partially suppress the wing vein phenotype of *D-stat*^{HJ} (48) and that *D-stat*^{HJ} and *D-stat*⁶³⁴⁶ can partially suppress the lethality (48) and melanotic-tumor formation (16), respectively, caused by *hop*^{Tum-l}. In conclusion, our results indicate that both Hop^{T42} and Hop^{Tum-l} directly hyperactivate D-Stat in vitro and in vivo.

DISCUSSION

The *hop*^{Tum-l} and *hop*^{T42} mutations hyperactivate Jak-Stat pathways. In this paper, we have documented that hyperactivation of a *Drosophila* Jak-Stat pathway causes hematopoietic and other developmental defects. We have provided biochemical and genetic evidence to verify that both the Hop Jak kinase and the D-Stat protein play direct roles in the phenotypes observed. First, we showed that both Hop^{Tum-l} and the newly identified Hop^{T42} mutant proteins hyperphosphorylated D-Stat when the proteins were coexpressed in insect cells. The hyperphosphorylated D-Stat protein displayed increased DNA binding activity to the D-Stat consensus recognition element, indicating that Hop^{Tum-l} and Hop^{T42} do indeed hyperactivate D-Stat. These results provide the first direct biochemical evidence for the hyperactivity of the Hop^{Tum-l} and Hop^{T42} proteins. Second, we showed that a similar mutation introduced into murine Jak2(E665K) caused increased tyrosine phosphorylation of Jak2 and increased activation of its substrate, Stat5. This experiment demonstrates the functional conservation between *Drosophila* and mammalian Jak kinases. Third, we dem-

FIG. 7. Model for the role of Hop Jak kinase pathways in *Drosophila* hematopoiesis. Hop plays a role in both proliferative and nonproliferative regulatory pathways. In contrast, the Hop–D-Stat pathway contributes to nonproliferative signaling, leading to the differentiation, aggregation, and/or melanization of hemocytes in *hop*^{Tum-l} and *hop*^{T42} mutants.

onstrated genetically that a reduction in D-Stat activity partially suppresses the lethality and melanotic-mass formation observed in *hop*^{Tum-l} and *hop*^{T42} mutants. These data indicate that the Jak-Stat pathway is hyperactivated in vivo by the *hop*^{Tum-l} and *hop*^{T42} mutations. Fourth, we found that hyperactivation of the Hop Jak kinase can be caused by an amino acid substitution in more than one domain of the protein.

We cannot rule out the possibility that Hop^{T42} and Hop^{Tum-l} affect other Hop-related pathways in addition to the Hop–D-Stat pathway (Fig. 7). Indeed, it is reasonable to expect, based on mammalian-cytokine studies, that the Hop Jak kinase participates in multiple regulatory pathways. Mammalian Jak kinases are thought to play a direct role in the phosphorylation and activation of other signal transduction pathways, including Ras-related pathways, through an interaction with SHC (15), Raf (45), or PI3 kinase (3, 30).

We propose the following model to describe the roles of Hop and D-Stat in larval hematopoiesis (Fig. 7). The Hop Jak kinase is involved in multiple signaling pathways in larval plasmotocytes and regulates the response to both proliferative and differentiative signals. The Hop–D-Stat pathway is not required for proliferation of the hemocytes but instead regulates the expression of genes involved in other hematopoietic functions. These functions could include the differentiation of plasmotocytes into lamellocytes and/or the expression of cell surface molecules, some of which are altered in *hop*^{Tum-l} mutants (31).

The *Drosophila* mutants suggest a role for Jak kinases in human disease. The first documentation that a mutant Jak kinase can cause abnormalities in hemocyte proliferation and differentiation came from earlier findings that the dominant *Tum-l* mutation was allelic to *hop* (11) and that *hop* encodes a Jak kinase (5). The data presented here further demonstrate the similarities between *Drosophila* and mammalian Jak kinases, as the equivalent mutation in the JH2 domains of a *Drosophila* and a murine Jak kinase hyperactivates both proteins. These findings suggest that mutations in components of Jak kinase pathways could play a role in mammalian hematopoietic disorders. Supporting this view, loss-of-function mutations in Jak3 have been implicated in severe combined immune deficiency (27, 34).

It is also possible that hyperactivation of mammalian Jak kinases can contribute to leukemogenesis. One study found that Jak2 was constitutively activated in leukemic cells from patients with acute lymphoblastic leukemia (29). The specific blocking of Jak2 activity in these cells inhibited leukemic-cell growth (29). However, a causal link between Jak kinase hyperactivation and mammalian blood cell abnormalities has not been firmly established. Based on the observation that Hop and murine Jak2 can both be hyperactivated by an equivalent mutation in the Jak kinase JH2 domain, we tested whether

murine Jak2(E665K) could transform a growth factor-dependent cell line, Ba/F3, to factor independence. Our preliminary results indicate that stable cell lines expressing Jak2(E665K) still required IL-3 for growth. However, it remains to be seen whether these cells now have a reduced requirement for IL-3 and whether such requirements are temperature sensitive. Further, the biological consequences of the increased Stat activation caused by Jak2(E665K) remain to be determined. To address these issues, we are currently attempting to generate transgenic mice that carry the Jak2(E665K) mutation.

Role of the Jak kinase JH2 domain. Members of the Jak kinase family are structurally different from other cytoplasmic protein tyrosine kinases and contain multiple protein domains that are conserved among Jak kinase family members. With the exception of the JH1 (kinase) domain, however, the functions of these domains have not been defined. The JH2 domain has similarities to known tyrosine kinase domains but does not contain several amino acid residues that are required for catalytic activity (10).

Our results with the Δ JH2 construct suggest that the JH2 domain of the Hop Jak kinase is required for Hop catalytic activity. Hop(Δ JH2) was not autophosphorylated and did not activate D-Stat in cotransfected Schneider cells. Therefore, it cannot serve solely as a negative regulatory domain for Jak activation, as is the case for a kinase-like domain in the cell surface receptor for atrial natriuretic peptide (6). It is possible that the JH2 domain serves primarily as an activation domain and that the E695K substitution in Hop^{T42} could cause Hop Jak kinase hyperactivation in several different ways. For example, the mutant JH2 domain might have an increased affinity for another protein, such as the receptor or D-Stat. It is also possible that the mutation additionally blocks the function of a negative regulatory protein. For example, the activities of mammalian Jak kinases are regulated in part by the Shp1 tyrosine phosphatase (21). The Hop^{T42} protein might be more resistant to dephosphorylation by a functional counterpart of this phosphatase, causing an increase in the catalytic activity of the kinase. Finally, it is not clear whether the mechanisms leading to the hyperactivation of Hop^{T42} and Hop^{Tum-1} are similar. We expect that a detailed mutational analysis of the *hop* gene, which is feasible for *Drosophila*, will resolve these questions.

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