The EMBO Journal vol.14 no.7 pp.1412–1420, 1995

An amino acid substitution in the *Drosophila* $hop^{Tum\text{-}l}$ Jak kinase causes leukemia-like hematopoietic defects

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Proteins of the Jak family of non-receptor kinases play important roles in mammalian hematopoietic signal transduction. They mediate the cellular response to a wide range of cytokines and growth factors. A dominant mutation in a *Drosophila* Jak kinase, $hopscotch^{Tumorous\text{-}lethal}$ ($hop^{Tum\text{-}l}$), causes hematopoietic defects. Here we conduct a molecular analysis of $hop^{Tum\text{-}l}$. We demonstrate that the $hop^{Tum\text{-}l}$ hematopoietic phenotype is caused by a single amino acid substitution of glycine to glutamic acid at residue 341. We generate a true revertant of the $hop^{Tum\text{-}l}$ mutation, in which both the molecular lesion and the mutant hematopoietic phenotype revert back to wild type. We also examine the effects of the G341E substitution in transgenic flies. The results indicate that a mutant Jak kinase can cause leukemia-like abnormalities.

Key words: *Drosophila*/hematopoiesis/$hop^{Tum\text{-}l}$/Jak kinase

Introduction

The fruit fly *Drosophila melanogaster* produces several types of hemocytes (reviewed in Rizki, 1978; Hoffman *et al.*, 1979). Spherical, phagocytic cells termed plasmatocytes serve a number of functions, including defense and cell-scavenging roles (Rizki and Rizki, 1984; Abrams *et al.*, 1992), and the secretion of anti-bacterial peptides (Hultmark, 1993) and extracellular matrix components (Fessler and Fessler, 1989). Plasmatocytes normally comprise ~90% of the circulating hemocytes in larvae (Rizki, 1957), and they are apparently the only circulating blood cells in the embryo (Tepass *et al.*, 1994). At the end of the larval stage of development, a significant number of plasmatocytes terminally differentiate into disc-like cells termed lamellocytes. Lamellocytes participate in encapsulation reactions. Another type of hemocyte is the crystal cell (Rizki, 1978) or oenocyte (Gupta, 1985), which contains crystalline inclusions. These inclusions are believed to be comprised of phenol oxidase precursors, which when released cause the melanization of encapsulated tissues (Rizki and Rizki, 1984). The larval precursors of circulating plasmatocytes and crystal cells are formed in the 'lymph gland', a set of 4–6 lobe pairs located at the anterior end of the dorsal vessel (Hoffman *et al.*, 1979).

The proliferation and differentiation of plasmatocytes are strongly affected by the $hopscotch^{Tumorous\text{-}lethal}$ ($hop^{Tum\text{-}l}$) mutation (Hanratty and Ryerse, 1981). $hop^{Tum\text{-}l}$ is a dominant, temperature-sensitive mutation (Corwin and Hanratty, 1976; Hanratty and Ryerse, 1981). At all culture temperatures, $hop^{Tum\text{-}l}$ plasmatocytes overproliferate, and can be present in concentrations 5–20 times that of wild-type plasmatocytes (Silvers and Hanratty, 1984; Zinyk *et al.*, 1993). At high temperatures, a substantial portion of plasmatocytes prematurely differentiate into lamellocytes. At these temperatures, many plasmatocytes and lamellocytes also have abnormal cell surfaces (Nappi and Silvers, 1984), and aggregate into large masses, which become melanized. The viability of hemizygous $hop^{Tum\text{-}l}$ males is drastically reduced at high, tumor-permissive temperatures, although the primary cause of lethality is uncertain. Crystal cells appear to be unaffected by the $hop^{Tum\text{-}l}$ mutation (Silvers and Hanratty, 1984). Complementation studies using radiation-induced revertants of $hop^{Tum\text{-}l}$ indicated that the mutation is in the *hop* locus (Hanratty and Dearolf, 1993). hop^{+} product is required for a number of developmental processes, including the proliferation of diploid imaginal precursor cells, the correct establishment of embryonic segmentation, and male fertility (Geer *et al.*, 1983; Perrimon and Mahowald, 1986). hop^{+} transcripts are expressed in a range of tissues and cell types, and are uniformly distributed during early embryogenesis (Binari and Perrimon, 1994).

The wild-type *hop* gene has been cloned, and encodes a putative member of the Jak family of non-receptor tyrosine kinases (Binari and Perrimon, 1994). These kinases are unique in structure, as they contain both a tyrosine kinase and a kinase-like domain, and lack strong SH2 and SH3 domains (Wilks and Harpur, 1994). The overall identity of Hop to the mammalian kinases Jak-1 (Wilks *et al.*, 1991), Jak-2 (Harpur *et al.*, 1992), Jak-3 (Johnston *et al.*, 1994; Witthuhn *et al.*, 1994) and Tyk-2 (Firmbach-Kraft *et al.*, 1990) ranges from 37 to 40% in the kinase domain, from 21 to 27% in the kinase-like domain, and from 19 to 23% in the remainder of the protein (Binari and Perrimon, 1994; H.Luo and C.Dearolf, unpublished observations).

Jak kinases play an important role in mammalian hematopoietic signal transduction pathways (reviewed in Wilks and Harpur, 1994). Jak kinases have recently been shown to mediate the cellular response to a large number of cytokines and growth factors, including interferons (IFNs) α and γ (Velazquez *et al.*, 1992; Watling *et al.*, 1993), interleukins (IL)-2, -3, -4, -6 and -11 (Silvennoinen *et al.*, 1993; Johnston *et al.*, 1994; Luetticken *et al.*, 1994; Stahl *et al.*, 1994; Witthuhn *et al.*, 1994; Yin *et al.*, 1994), erythropoietin (Witthuhn *et al.*, 1993), granulocyte colony-stimulating factor (Fukunaga *et al.*, 1993), granulocyte-macrophage colony-stimulating factor (Quelle *et al.*,

1994), epidermal growth factor (Shuai *et al.*, 1993), growth hormone (Argetsinger *et al.*, 1993) and prolactin (Dusanter-Fourt *et al.*, 1994). Most Jak kinases are expressed ubiquitously, although Jak-3 expression is more restricted (Johnston *et al.*, 1994; Witthuhn *et al.*, 1994). The pathways in which Jak kinases are involved are best understood for the IFNs (Shuai *et al.*, 1993). Stimulation by IFN α or γ leads to the activation of Jak-1 and Tyk-2, or Jak-1 and Jak-2, respectively, and to the phosphorylation of signal transducer and activator of transcription (STAT) proteins. The STAT proteins then translocate to the nucleus and form active transcription factor complexes (Darnell *et al.*, 1994).

A number of questions concerning the regulation of hematopoiesis by Jak kinases need to be answered. Still unknown are the precise roles Jak kinases play in the Jak-STAT signaling pathways, the similarities of other cytokine- and growth factor-induced pathways to those of the IFNs, and the effects of mutant Jak kinases on hematopoiesis. *Drosophila* hemocytes offer an alternative genetic system to address these questions. Similarities between *Drosophila* and mammalian hematopoietic genes have been identified. For example, both mammalian Jak kinases and the *Drosophila hop* Jak kinase are involved in the regulation of hematopoiesis. In addition, the intracellular domain of the *Drosophila Toll* receptor is similar to that of the IL-1 receptor (Schneider *et al.*, 1991). Dominant *Toll* mutations can also cause melanotic mass formation (Gerttula *et al.*, 1988). Further, the *Drosophila* Dorsal-related immunity factor (Dif) contains a *rel* homology domain (Ip *et al.*, 1993), and recognizes similar promoter elements as the Rel-containing protein NF-κB (Kieran *et al.*, 1990; Nolan *et al.*, 1991). Dif regulates the transcription of *Drosophila* anti-bacterial genes, in hemocytes and in fat body, whereas NF-κB regulates the transcription of mammalian lymphoid genes (Brasier *et al.*, 1990; Nonaka and Huang, 1990). Both Dif and NF-κB are themselves regulated at the level of nuclear transport (Davis *et al.*, 1991; Haskill *et al.*, 1991; Ip *et al.*, 1993).

In this paper, we characterize the molecular lesion in *hop*$^{Tum\text{-}l}$. We identify an amino acid substitution of glycine to glutamic acid at residue 341 (G341E) of the predicted Hop$^{Tum\text{-}l}$ protein. We take two approaches to verify that this substitution causes the *hop*$^{Tum\text{-}l}$ hematopoietic phenotype. First, we generate a true ethylmethane sulfonate (EMS) revertant of *hop*$^{Tum\text{-}l}$, in which both the molecular lesion and the hematopoietic phenotype revert back to wild type. Second, we create transgenic flies, in which the strong mutant phenotype results from constitutive expression of *hop*$^{Tum\text{-}l}$, but not *hop*$^{+}$ cDNA. Our results provide the first example of leukemia-like defects caused by a mutant Jak kinase.

Results

Generation of hop$^{T\text{-}r7}$*, a true revertant of hop*$^{Tum\text{-}l}$

To identify amino acid residues in *hop*$^{Tum\text{-}l}$ that are critical for the mutant phenotype, we conducted a chemical mutagenesis screen for revertants of *hop*$^{Tum\text{-}l}$. We mutagenized *hop*$^{Tum\text{-}l}$ hemizygous males with EMS and screened for revertants that lacked melanotic mass formation (see Materials and methods). Three independent revertants of the *hop*$^{Tum\text{-}l}$ locus were recovered, out of 940 mutagenized X chromosomes. We were unable to separate each revertant from *hop*$^{Tum\text{-}l}$ by meiotic recombination (data not shown), indicating that the induced mutations lie within or very close to the *hop*$^{Tum\text{-}l}$ locus. One of these revertants, *hop*$^{Tum\text{-}l\ revertant\ \#7}$ (*hop*$^{T\text{-}r7}$), is viable at both high and low temperatures. *hop*$^{T\text{-}r7}$/Basc females were mated to Basc males, and the progeny were cultured at 28°C. More than 100% of the expected number of revertant males survived to adulthood, relative to Basc male siblings (Table I). This survival rate is comparable to that of *hop*$^{+}$ controls. Under similar conditions, only 7% of the expected number of *hop*$^{Tum\text{-}l}$ males survived (Table I). Both *hop*$^{T\text{-}r7}$ and *hop*$^{Tum\text{-}l}$ males are fully viable at 17°C (Table I).

We determined by three additional criteria that the hematopoietic defects are eliminated in *hop*$^{T\text{-}r7}$ revertants. First, melanotic mass formation in *hop*$^{T\text{-}r7}$ revertants is eliminated (Figure 1A; Table II). Female adults heterozygous for *hop*$^{Tum\text{-}l}$, *hop*$^{T\text{-}r7}$, or homozygous for *hop*$^{+}$, were cultured at 29°C and examined for the presence of melanotic masses. *hop*$^{Tum\text{-}l}$ mutants cultured at high temperatures are notable for the formation of black, melanotic masses (Figure 1C; Table II; Hanratty and

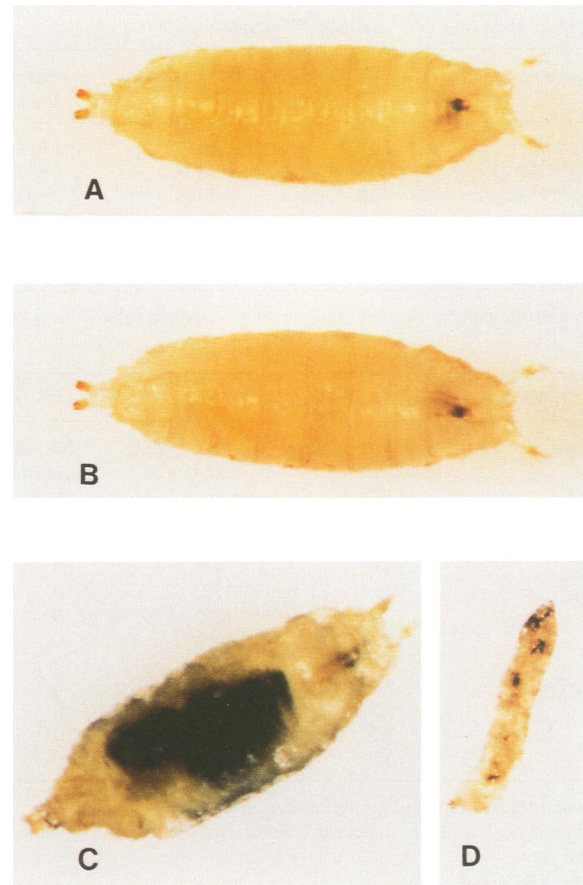

Fig. 1. *hop*$^{T\text{-}r7}$ and *hop*$^{Tum\text{-}l}$ prepupae and larvae, cultured at 28°C. (**A**) A hemizygous *hop*$^{T\text{-}r7}$ prepupa. *hop*$^{T\text{-}r7}$ revertants lack melanotic masses, comparable to wild type. The black mouth hooks can be seen in the anterior region of the prepupae. (**B**) A prepupa containing two genomic copies of the transformed pCaSpeR Act/*T-r7* construct. Melanotic masses are not present. (**C**) A hemizygous *hop*$^{Tum\text{-}l}$ prepupa, containing a very large melanotic mass. (**D**) A first instar larva, transgenic for the pCaSpeR Act/*Tum-l* construct. The larva contains multiple, small melanotic masses, and did not develop past this stage. In each picture, the anterior faces towards the right.

Table I. Survival of $hop^{T\text{-}r7}$ and $hop^{Tum\text{-}l}$ adults

Chromosome tested	Temperature (°C)	Total adults	Heterozygous females	Basc females	Hemizygous males	Basc males	% Hemizygous males[a]
y v hop^{+} *m*	28	1972	518	449	555	450	>100
y v $hop^{T\text{-}r7}$ *m*	28	1823	577	429	429	388	>100
y v $hop^{Tum\text{-}l}$ *m*	28	1415	502	453	31	429	7.2
y v hop^{+} *m*	17	1812	588	406	512	306	>100
y v $hop^{T\text{-}r7}$ *m*	17	3149	1052	749	814	534	>100
y v $hop^{Tum\text{-}l}$ *m*	17	2891	1067	627	609	588	>100

[a]Represents the percent of hemizygous males recovered relative to Basc male siblings.

Table II. Melanotic mass formation in $hop^{Tum\text{-}l}$ and revertant $hop^{T\text{-}r7}$ females

Genotype	Temperature (°C)	No. of adults examined	No. with melanotic mass[a]	No. with melanotic mass size			No. with other defects[b]	% of normal adults
				+	++	+++		
y v $hop^{Tum\text{-}l}$ *m*	29	50	50	0	12	38	ND	0
Basc	17	47	10	9	1	0	ND	79
y v $hop^{T\text{-}r7}$ *m*	29	132	0				4	97
Basc	26	135	0				0	100
	22	116	0				5	96
	17	204	0				10	95
y v hop^{+}*m*	29	89	0				4	96
Basc	26	114	0				0	100
	22	134	0				2	99
	17	105	0				0	100

[a]Melanotic masses reported are those seen in undissected adults.
[b]Other defects, detected in dissected females, include ovary discoloration, possible remnants of larval trachea or a single black fleck approximately pinpoint size.
ND, not determined.

Table III. Transplantation of $hop^{Tum\text{-}l}$ and revertant $hop^{T\text{-}r7}$ lymph glands

Genotype of male donor	Hosts		No. with abdominal melanotic mass	No. with melanotic legs				Size of injected lymph gland tissue recovered			
	No. Total	No. Bloated		0	1–2	3–4	5–6	+++	++	+	none
y v $hop^{Tum\text{-}l}$ *m*	35	18	34	1	0	3	31	27	5	2	1
y v $hop^{T\text{-}r7}$ *m*	37	0	0	36	1	0	0	0	0	7	30

Ryerse, 1981). These masses consist of aggregated hemocytes, either free in the hemolymph or attached to other tissues, particularly the gastric cecae. These masses are not seen in $hop^{T\text{-}r7}$ or in hop^{+} females, at all temperatures tested. Occasional non-hematopoietic defects are seen in both $hop^{T\text{-}r7}$ and hop^{+} females (Table II).

Second, $hop^{T\text{-}r7}$ lymph glands have lost the ability to form transplantable neoplasms (Table III). $hop^{Tum\text{-}l}$ and $hop^{T\text{-}r7}$ lymph gland tissues were injected into Oregon R adult females, which were then cultured at 29°C. Hosts were examined 3–6 days later. The $hop^{Tum\text{-}l}$ lymph gland caused significant defects in the wild-type hosts. Melanotic masses appeared in the abdominal cavity and/or in leg joints of 34/35 of the injected hosts. Bloating, which is often associated with hosts containing rapidly growing neoplastic tissue (Gateff and Schneiderman, 1969), was observed in 18/35 hosts. In addition, large pieces of injected lymph gland tissue could be dissected out of a majority of the hosts. In contrast, bloating was not observed in the hosts injected with $hop^{T\text{-}r7}$ lymph glands, the injected tissue was small or not found upon re-dissection, and only one small melanotic mass was detected out of 37 injected hosts. This latter defect may be due to injury caused by the injection procedure.

Third, circulating hemocyte concentrations in $hop^{T\text{-}r7}$ larvae are comparable to those of wild-type larvae (Figure 2). We determined plasmatocyte and lamellocyte concentrations from mid–late third instar larvae grown at 28°C. In wild-type larvae at this stage, lamellocytes comprise <5% of the circulating hemocyte population (Figure 2B). $hop^{Tum\text{-}l}$ hemocytes show a mutant pattern (Figure 2; Silvers and Hanratty, 1984). They overproliferate and differentiate prematurely, with ~50% of the circulating hemocyte population composed of lamellocytes. In contrast, the $hop^{T\text{-}r7}$ hemocyte concentration and percentage of differentiated hemocytes resemble those of Oregon R larvae. Therefore, we conclude that the $hop^{T\text{-}r7}$ hematopoietic phenotype is comparable with that of wild-type flies.

Amino acid substitutions in the $Hop^{Tum\text{-}l}$ *protein*

We previously identified the *Tumorous-lethal* mutation as an allele of *hop* by complementation studies between γ-radiation-induced revertants of $hop^{Tum\text{-}l}$ and recessive *hop*

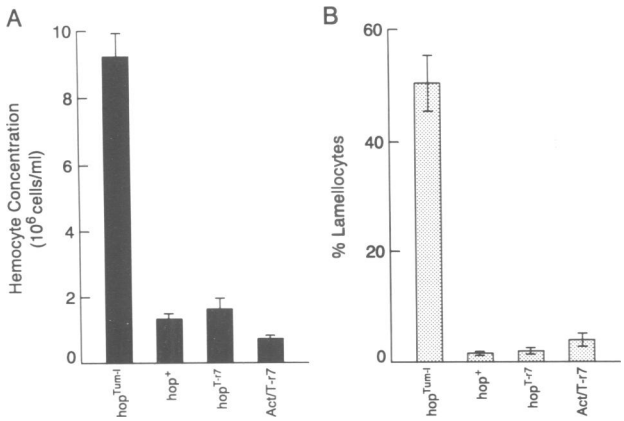

Fig. 2. Circulating hemocytes in mid−late third instar larvae, cultured at 28°C. (**A**) The total concentration of plasmatocytes and lamellocytes, given in 10^6 cells/ml. (**B**) The percentage of lamellocytes, out of the total plasmatocytes and lamellocytes determined. The genotypes examined include hemizygous *hop*$^{Tum\text{-}l}$, wild-type Oregon R, hemizygous *hop*$^{T\text{-}r7}$ and two genomic copies of the transformed pCaSpeR Act/*T-r7* construct. For each genotype, hemocyte counts were made from 10 larvae, with the mean and SE shown. Both the total concentration and percent lamellocytes are significantly higher in *hop*$^{Tum\text{-}l}$ larvae compared with each of the other genotypes ($P < 0.01$ by *t*-test analysis).

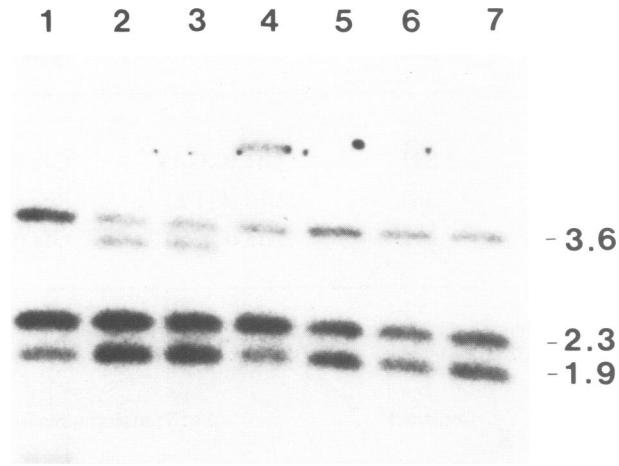

Fig. 3. Southern analysis of γ-radiation-induced revertants of *hop*$^{Tum\text{-}l}$. Lanes 1–5, genomic DNA from revertant *hop*$^{Tr\text{-}3}$, *hop*$^{Tr\text{-}1}$, *hop*$^{Tr\text{-}2}$, *hop*$^{Tr\text{-}4}$ and *hop*$^{Tr\text{-}5}$ heterozygotes, all balanced over Basc; lane 6, *hop*$^{Tum\text{-}l}$/ Basc DNA; lane 7, Basc/Basc DNA. Genomic DNA was cut with *Sal*I and probed with *hop*$^{+}$ cDNA. Sizes of bands detected in the control Basc and *hop*$^{Tum\text{-}l}$/ Basc chromosomes are given. Revertants *r1*−*r4* each show a restriction site polymorphism, whereas *hop*$^{Tum\text{-}l}$ and revertant *r5* show a similar pattern to Basc. Revertants *r1* and *r2* show a similar alteration, and most likely represent multiple isolates of the same reversion event. The *r5* revertant may contain a lesion in the *hop*$^{Tum\text{-}l}$ gene outside of the transcribed region, or may have a deletion or rearrangement too small to be detected by Southern analysis.

alleles (Hanratty and Dearolf, 1993). We suspected that the molecular lesion in *hop*$^{Tum\text{-}l}$ is a point mutation in the protein-coding region, and that the revertants had undergone a chromosomal rearrangement or deletion in the gene. To test this possibility, we performed a Southern analysis of genomic DNA isolated from *hop*$^{Tum\text{-}l}$, five γ-ray-induced revertants of *hop*$^{Tum\text{-}l}$ and Basc controls using *hop*$^{+}$ cDNA (Binari and Perrimon, 1994) as a probe (Figure 3). The results indicate that four of the five revertants show a restriction fragment polymorphism, and that *hop*$^{Tum\text{-}l}$ and Basc have a similar restriction fragment pattern in the transcribed region of the *hop* gene, consistent with our hypothesis.

To identify the molecular basis of the *hop*$^{Tum\text{-}l}$ mutation, we sequenced the protein-coding regions of *hop*$^{Tum\text{-}l}$ and *hop*$^{T\text{-}r7}$. We isolated total RNA from third instar larvae of hemizygous males and conducted reverse transcription-polymerase chain reaction (RT-PCR). The amplified PCR products were then either directly sequenced, or first subcloned into pBluescript SK(+) and subsequently sequenced. In the latter case, at least two independent clones were sequenced to eliminate possible artifacts created by PCR.

We found three amino acid changes in Hop$^{Tum\text{-}l}$ as compared with the published Hop^{+} sequence (Figure 4). These amino acid changes include glycine to glutamic acid at residue 341, valine to phenylalanine at residue 365, and alanine to glycine at residue 592. One of these amino acid substitutions, G341E, is reverted back from glutamic acid (GAA) to wild-type glycine (GGA) in *hop*$^{T\text{-}r7}$ (Figure 4). We also sequenced *hop*$^{+}$ cDNA obtained from a *yellow* (*y*) control stock. This stock contains a glycine amino acid at residue 341, but is identical to *hop*$^{Tum\text{-}l}$ at residues 365 and 592 (Figure 4). The *hop*$^{+}$ sequence of the *y* stock is identical to that of *hop*$^{T\text{-}r7}$. These results suggest that the *hop*$^{Tum\text{-}l}$ mutation is caused by the G341E substitution, and that the *hop*$^{T\text{-}r7}$ revertant has undergone a true reversion of the *hop*$^{Tum\text{-}l}$ mutation back to wild type. It is likely that the differences observed in the original *hop*$^{+}$ cDNA clone at residues 365 and 592 represent inconsequential polymorphisms.

The G341E substitution in Hop$^{Tum\text{-}l}$ occurs in the amino-terminal third of the protein, outside of the predicted kinase (JH1) and kinase-like (JH2) domains (Wilks and Harpur, 1994). The corresponding amino acid in the mammalian Jak kinases is not obvious, although based on sequence alignments it occurs in or amino terminal to the JH4 domain (data not shown).

Transgenic analysis

We then generated transgenic flies to verify the significance of the G341E substitution. Because the regulatory regions necessary for endogenous *hop* transcription are not yet known, we used the constitutive cytoplasmic actin5C promoter (Vigoreaux and Tobin, 1987) to express the *hop*$^{Tum\text{-}l}$ and the *hop*$^{T\text{-}r7}$ coding regions. These two cDNAs are identical, with the exception of the single difference of Glu (GAA) in *hop*$^{Tum\text{-}l}$, or Gly (GGA) in *hop*$^{T\text{-}r7}$, at codon 341. The results are summarized in Figure 5. We recovered two independent pCaSpeR Act/*T-r7* transformant lines, each of which has an insertion on the second chromosome. Both transformant stocks are homozygous viable and fertile. The constitutive expression of *hop*$^{T\text{-}r7}$ does not affect the viability of these transgenic flies (Table IV), and two copies of the pCaSpeR Act/*T-r7* construct do not result in melanotic mass formation (Figure 1B) or in abnormal circulating hemocyte concentrations (Figure 2).

To verify that the *hop*$^{T\text{-}r7}$ cDNA is indeed expressed in the transgenic stocks, we examined whether the pCaSpeR

Amino Acid Residue	Hop+ (cDNA library)	Hop+ (*y* stock)	Hop^{Tum-l}	Hop^{T-r7}
341	Gly (GGA)	Gly (GGA)	Glu (GAA)	Gly (GGA)
365	Val (GTT)	Phe (TTT)	Phe (TTT)	Phe (TTT)
592	Ala (GCC)	Gly (GGC)	Gly (GGC)	Gly (GGC)

Fig. 4. Differences among the *hop*$^{+}$, *hop*$^{Tum-l}$ and *hop*$^{T-r7}$ predicted amino acid sequences. The Hop^{+} sequence of a cDNA library clone was determined by Binari and Perrimon (1994).

Construct	# of Transformants Recovered	28°C Melanotic Mass Formation	Rescues Recessive *hop* Lethality
pCaSpeR Act/*T-r7*	2 stable lines	No	Yes
pCaSpeR Act/*Tum-l*	5 larval lethal lines	Yes	(No)

Fig. 5. Summary of P-element transformations.

Table IV. Survival of transgenic pCaSpeR Act/*T-r7* flies

Cross	Total progeny	Act/*T-r7* females	CyO females	Act/*T-r7* males	CyO males	% Act/*T-r7* progeny[a]
OreR×pCaSpeR Act/*T-r7* (1) / CyO	1111	316	260	280	255	116
OreR×pCaSpeR Act/*T-r7* (2) / CyO	1019	285	250	272	212	121

[a]Represents the percent of pCaSpeR Act/*T-r7* heterozygous progeny, relative to CyO siblings.

Act/*T-r7* transformant chromosome can rescue the recessive lethality of known *hop* alleles (Table V). Females heterozygous for the *hop* allele shown, balanced over Basc, were mated to males homozygous for pCaSpeR Act/*T-r7* on the second chromosome. In the absence of additional *hop*$^{+}$ genes, each recessive *hop* allele tested is completely lethal prior to the adult stage (Perrimon and Mahowald, 1986; Hanratty and Dearolf, 1993). One copy of the pCaSpeR Act/*T-r7* insertion rescues the lethality of the strong recessive *hop* alleles *L4* and *CIII*, and of the radiation-induced *hop*$^{Tum-l}$ revertants. Further, the transgenic chromosome rescues the male hemizygous sterility of the hypomorph *hop*^{msv1} (data not shown). Therefore, the pCaSpeR Act/*T-r7* insertions generate a functional and normal product.

Experiments with the pCaSpeR Act/*Tum-l* construct gave sharply contrasting results (Figures 1D and 5). We were unable to recover stable transformant stocks for the pCaSpeR Act/*Tum-l* construct, even though >2000 embryos were injected, and both parents and progeny were cultured at 17°C to minimize deleterious effects of the mutation. Nevertheless, five of the surviving adults produced progeny that are likely to represent individuals transformed with the pCaSpeR Act/*Tum-l* construct. After 2 weeks of culture at 17°C, the adults were shifted to 28°C and their progeny were allowed to develop at this temperature. At 28°C, some progeny from these five injected adults died as first instar larvae that have multiple internal melanotic masses (Figure 1D). This level of melanotic mass formation is comparable to that found in first instar larvae of hemizygous *hop*$^{Tum-l}$ males cultured at 28°C. We did not detect similar larvae in *hop*$^{+}$ controls or in pCaSpeR Act/*T-r7* lines at this temperature.

To verify the presence of the integrated pCaSpeR Act/*Tum-l* construct in these dying progeny, we pooled 13 such progeny obtained, isolated genomic DNA and performed PCR to amplify the sequence covering the codon at residue 341. PCR primers were chosen (see Materials and methods) so that the PCR amplification products of the inserted pCaSpeR Act/*Tum-l* cDNA construct and of the endogenous genomic *hop*$^{+}$ DNA would have distinct sizes. Amplification of the pCaSpeR Act/*Tum-l* construct would yield a 441 bp product. In contrast, amplification of the endogenous genomic *hop*$^{+}$ DNA would yield a product of ~1.4 kb, because the primers hybridize to genomic DNA that is separated by two intron regions (Binari and Perrimon, 1994). We obtained a 441 bp PCR product from the reaction (Figure 6), suggesting the presence of the inserted pCaSpeR Act/*Tum-l* construct. We then recovered and directly sequenced this reaction product, and observed the presence of the mutant *hop*$^{Tum-l}$ sequence (data not shown). We therefore conclude that the G341E amino acid substitution in *hop*$^{Tum-l}$ is responsible for the mutant hematopoietic phenotype, and that the constitutive expression of the *hop*$^{Tum-l}$ protein causes larval lethality and melanotic mass formation in these transgenic flies. It is likely that the lethality of pCaSpeR Act/*Tum-l* transformed larvae, even when cultured at 17°C, is caused by the deleterious effects of constitutive *hop*$^{Tum-l}$ expression in non-hematopoietic tissues.

Table V. Rescue of *hop* recessive lethality by pCaSpeR Act/*T-r7*

hop allele tested	Total progeny	*hop* males	Basc males	% *hop* males
hop^{CIII}	586	152	103	>100
hop^{L4}	698	189	92	>100
$hop^{T\text{-}r1}$	534	143	97	>100
$hop^{T\text{-}r3}$	574	146	110	>100
$hop^{T\text{-}r4}$	445	118	103	>100
$hop^{T\text{-}r5}$	493	142	84	>100

Discussion

In this paper, we have demonstrated that the hematopoietic defects in $hop^{Tum\text{-}l}$ mutants are caused by the G341E amino acid substitution. This conclusion is supported by several lines of evidence. First, the $hop^{Tum\text{-}l}$ larval cDNA sequence differs from two wild-type *hop* sequences at this codon. Second, constitutive expression of the mutant $hop^{Tum\text{-}l}$ cDNA, but not a wild-type cDNA, leads to the formation of melanotic masses and to lethality in transgenic flies. Third, both the mutant phenotype and the mutation at codon 341 are reverted to wild type in $hop^{T\text{-}r7}$.

Our results provide the first evidence that a point mutation in a Jak kinase can misregulate hemocyte proliferation and differentiation. The defects in $hop^{Tum\text{-}l}$ plasmatocyte proliferation have similarities to those of mammalian leukemias. In both cases hemocytes overproliferate, the overproliferation is clonal in origin and a subset of hematopoietic lineages is affected. At present, associations between mammalian leukemias and Jak kinase mutations have not been reported. It is possible that links between Jak kinases and leukemia will be found, but have not yet been detected because of the novelty of this protein family. It is also possible that mutant Jak kinases become transforming only in combination with mutations in other regulatory proteins.

The $hop^{T\text{-}r7}$ revertant

$hop^{T\text{-}r7}$ is a true revertant of $hop^{Tum\text{-}l}$. Phenotypically, $hop^{T\text{-}r7}$ revertants resemble wild-type rather than $hop^{Tum\text{-}l}$ mutants in each of the assays tested, including hemizygous viability, melanotic mass formation, circulating blood cell concentrations and lymph gland transplantations. Further, the transgenic experiments demonstrate that the $hop^{T\text{-}r7}$ cDNA produces a functional and normal product that rescues the strong mutant phenotype of known *hop* recessive alleles. Finally, the $hop^{T\text{-}r7}$ sequence is identical to that of hop^{+} in the *y* stock.

It is interesting that we obtained a true revertant of $hop^{Tum\text{-}l}$ from a small mutagenesis screen. Also, the overall frequency of $hop^{Tum\text{-}l}$ revertants recovered, 3/940 potentially mutagenized X chromosomes, is high. The reason for this recovery rate is not certain. It is possible that the *hop* locus is highly mutable. Alternatively, mutations in the gene may lead to a scorable phenotype at a high frequency, enhancing the recovery of mutant strains. Consistent with these hypotheses, >40 recessive *hop* alleles and two dominant alleles have been recovered from a variety of mutagenesis screens, in which selection was conducted for recessive lethality, for male sterility, for zygotic lethals having a maternal effect, for melanotic mass formation, or for loss of $hop^{Tum\text{-}l}$ melanotic mass formation (Lefevre, 1971; Corwin and Hanratty, 1976; Geer *et al.*, 1983; Perrimon and Mahowald, 1986; Lindsley and Zimm, 1992; Hanratty and Dearolf, 1993; and W.P.Hanratty and C.R.Dearolf, unpublished observations).

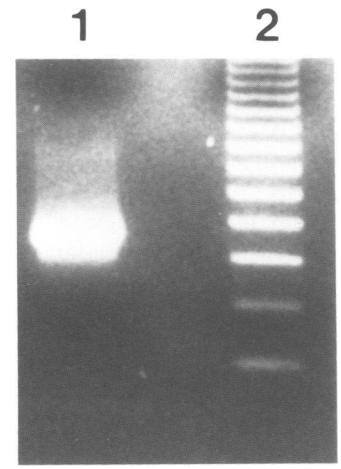

Fig. 6. PCR amplification of the pCaSpeR Act/*Tum-l* construct in transformed larvae. Lane 1, PCR amplification product. Template DNA was isolated from dying larvae, cultured at 28°C, that were suspected to contain the pCaSpeR Act/*Tum-l* construct. A 441 bp band, indicative of the pCaSpeR Act/*Tum-l* construct, is generated. Lane 2, 123 bp DNA ladder.

Cell autonomy of the $hop^{Tum\text{-}l}$ mutation

The $hop^{Tum\text{-}l}$ mutation causes several types of hematopoietic defects. The overproliferation of $hop^{Tum\text{-}l}$ plasmatocytes occurs at all culture temperatures tested, whereas the premature differentiation of plasmatocytes into lamellocytes, changes in hemocyte cell surface molecules, and the aggregation and melanization of hemocytes are temperature sensitive (Hanratty and Ryerse, 1981; Nappi and Silvers, 1984; Zinyk *et al.*, 1993). The overproliferation of $hop^{Tum\text{-}l}$ plasmatocytes appears to be cell autonomous. This conclusion is directly supported by the lymph gland transplantation experiments reported here (Table III) and previously (Hanratty and Ryerse, 1981), in which $hop^{Tum\text{-}l}$ hematopoietic cells continue to overproliferate when placed in a wild-type environment. Therefore, we conclude that the overproliferation of $hop^{Tum\text{-}l}$ hemocytes is an intrinsic defect of the blood cells.

The primary cause of the other $hop^{Tum\text{-}l}$ hematopoietic defects at high temperatures, including abnormal cell surfaces, premature differentiation and melanotic mass formation, is not clear. It is possible that the $hop^{Tum\text{-}l}$ mutation autonomously affects several distinct regulatory pathways in *Drosophila* hemocytes. By this interpretation, the pathway that stimulates proliferation is activated at all temperatures, whereas the differentiation pathway becomes activated in mutants only at tumor-permissive temperatures. An alternative possibility is that this phenotype represents an immune response of hematopoietic cells to defects in other tissues. The hematopoietic immune response is activated as a defense mechanism to a variety of stimuli, including wounding, pathogens, parasites and heterospecific tissue implants (Rizki and Rizki, 1984). It has also been shown that hemocytes can respond to the abnormal cell surfaces of non-hematopoietic tissue in mutant larvae (Rizki and Rizki, 1974a,b). The gastric

cecae are frequently abnormal in $hop^{Tum\text{-}l}$ larvae grown at tumor-permissive temperatures (Hanratty and Ryerse, 1981). Therefore, defects in these or other cells may trigger the immune response. A similar rationale could be used to explain the appearance of melanotic masses following the transplantation of $hop^{Tum\text{-}l}$ lymph glands into wild-type hosts (Table III). In these experiments, the transplanted lymph glands produce a high number of hemocytes around the ovaries of their female hosts, leading to the extensive destruction of ovarian tissue (Hanratty and Ryerse, 1981). These defects in host tissue could then induce the formation of melanized masses.

$Hop^{Tum\text{-}l}$ as an activated Jak kinase

We propose that the G341E mutation causes the $Hop^{Tum\text{-}l}$ protein to become a constitutively activated kinase in hemocytes. Several molecular mechanisms can be envisioned to account for this activation. The amino acid substitution may impair or eliminate the function of a negative regulatory domain. For example, point mutations have been identified in non-catalytic domains of the non-receptor tyrosine kinases v-Src and c-Src that increase the transforming potential and/or protein kinase activity of the mutant proteins (Kato *et al.*, 1986; Catling *et al.*, 1994). These amino acids have been postulated to be components of negative regulatory domains that contribute to maintaining the Src kinase in an inactive state (Okada *et al.*, 1993; Superti-Furga *et al.*, 1993). It is possible that the amino-terminal region of $Hop^{Tum\text{-}l}$ contains important regulatory regions.

Alternatively, the amino acid substitution could enhance the interactions of a positive regulatory domain. It has been shown that mammalian Jak kinases associate with the membrane-proximal regions of cytokine and growth factor receptors, including the erythropoietin receptor (Witthuhn *et al.*, 1993), the growth hormone receptor (Argetsinger *et al.*, 1993) and gp130 (Narazaki *et al.*, 1994; Stahl *et al.*, 1994). $Hop^{Tum\text{-}l}$ could constitutively associate with the appropriate domains of membrane-bound receptors, resulting in the formation of an active complex, even in the absence of ligand binding. It is also possible that the $hop^{Tum\text{-}l}$ mutation causes the protein to adopt a novel conformation, resulting in an active Jak kinase that directly activates the downstream components of signaling pathways in plasmatocytes. Biochemical studies are needed to differentiate among these alternatives.

Genetic dissection of Drosophila hematopoiesis

Drosophila offers a powerful genetic system to study the regulation of hematopoiesis. A number of *Drosophila* mutations that cause hematopoietic abnormalities have been identified (Stark, 1919; Wilson, 1924; Wilson *et al.*, 1955; Sang and Burnet, 1963; Sparrow, 1974, 1978; Gateff, 1978; Hanratty and Ryerse, 1981; Gerttula *et al.*, 1988; Watson *et al.*, 1991; Lindsley and Zimm, 1992). In addition to $hop^{Tum\text{-}l}$, several of these genes have been characterized at the molecular level. The air^{8} gene encodes the *Drosophila* S6 ribosomal protein (Watson *et al.*, 1992; Stewart and Denell, 1993). The *Toll* gene encodes a receptor protein whose intracellular domain is similar to that of mammalian IL-1 receptors (Schneider *et al.*, 1991). The *l(3)mbn-1* gene has also been cloned and sequenced, although its function is unclear (Konrad *et al.*, 1994). Mutations in each of these genes cause the formation of melanotic masses in *Drosophila.*

These mutations can be used to identify other components of hematopoietic regulatory pathways. For example, we have identified the $awd^{K\text{-}pn}$ and *pn* mutations as second-site suppressors of the $hop^{Tum\text{-}l}$ phenotype (Zinyk *et al.*, 1993), and have recovered another mutation that both suppresses $hop^{Tum\text{-}l}$ and strongly enhances the recessive *hop* phenotype (C.Dearolf, unpublished observations). A thorough characterization of these mutations should further our understanding of the genetic regulation of hematopoiesis.

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 are described in Lindsley and Zimm (1992) and Hanratty and Dearolf (1993), and include *hopscotch* and *Tumorous-lethal* (*hop* and $hop^{Tum\text{-}l}$, 1-34.5), *yellow* (*y*, 1-0.0), *white* (*w*, 1-1.5), *vermillion* (*v*, 1-33.0) and *miniature* (*m*, 1-36.1). Both $hop^{Tum\text{-}l}$ and $hop^{T\text{-}r7}$ were maintained on *y v m* chromosomes, balanced over Basc or FM7a. The second chromosome balancer CyO was also utilized. The wild-type strain used was Oregon R. In each experimental cross conducted, care was taken to avoid the overcrowding of progeny. Lymph gland transplantations and larval hemocyte concentrations were performed as previously described (Hanratty and Ryerse, 1984; Zinyk *et al.*, 1993). The sizes of melanotic masses observed and transplanted lymph tissue recovered were classified as: + for small melanotic flecks or barely observable tissue pieces under the dissection microscope; ++ for slightly larger to moderate sizes; +++ for large melanotic masses and lymph tissue as large as the fragments originally injected.

Generation of $hop^{T\text{-}r7}$ revertant

y v $hop^{Tum\text{-}l}$ *m* males, grown at 17°C, were fed EMS according to the method of Lewis and Bacher, 1968, and mated to Basc females. *y v* $hop^{Tum\text{-}l}$ *(*) m*/Basc female progeny were individually mated to sibling Basc males, and the progeny cultured at 29°C. At 29°C, the $hop^{Tum\text{-}l}$ stock is hemizygous lethal. Adult progeny were simultaneously screened for either of two classes. First, progeny were examined for the presence of non-Basc males. These males were mated to Basc females, and the X chromosome re-tested for viability. Second, female *y v* $hop^{Tum\text{-}l}$ *(*) m/* Basc progeny were scored for the absence of melanotic masses. Such females were mated to Basc males and the mutagenized X chromosome tested for complementation with recessive *hop* alleles. Three $hop^{Tum\text{-}l}$ revertants were recovered, out of 940 potentially mutagenized X chromosomes.

Southern analysis

Genomic DNA was isolated from adult flies as previously described (Dearolf *et al.*, 1988). Southern analysis was carried out using standard protocols (Sambrook *et al.*, 1989). For each lane, 20 μg of DNA were digested with *Sal*I, electrophoresed in a 1% agarose gel, blotted onto nylon membrane and hybridized to a ^{32}P-labeled 5.1 kb zygotic hop^{+} cDNA (Binari and Perrimon, 1994). Signal was detected by autoradiography following 2 days of exposure at −70°C.

DNA sequencing and cDNA construction

Total RNA was isolated from hemizygous *y v* $hop^{Tum\text{-}l}$ *m*, *y v* $hop^{T\text{-}r7}$*m*, and *y* third instar larvae. To amplify the protein-coding region of the *hop* transcript, ~1 μg of total RNA was used in reverse transcription-PCR, using the Perkin Elmer Cetus GeneAmp RNA PCR kit. The complete coding region was amplified using three sets of PCR primers, covering the sequence from −45 bp relative to the predicted translation start site to the 20 bp immediately after the predicted translation stop codon (Binari and Perrimon, 1994). The three amplification products were ~800 bp, 1.55 kb and 1.4 kb in size. The first two fragments overlapped so that they both included the endogenous *Eco*RI restriction site at nucleotide 690. The second and third fragments both overlapped the *Eco*RI restriction site at nucleotide 2150. To facilitate later cloning procedures, we added unique *Xho*I or *Xba*I restriction enzyme sites to the primers that hybridized to the 5′ and 3′ ends of the cDNA,

respectively. We conducted both plasmid DNA sequencing of cloned PCR products, and direct sequencing of PCR products. For the sequencing of plasmid DNA, multiple clones were examined, so as to reduce the likelihood of PCR artifacts. For the direct sequencing of PCR products, the Promega fmol DNA sequencing system was used according to the manufacturer's instructions. To generate cDNA clones containing the complete predicted coding region, we utilized the internal *Eco*RI sites to ligate correct PCR amplification fragments.

Generation and sequence analysis of transgenic flies

hop$^{Tum\text{-}l}$ and revertant *hop*$^{T\text{-}r7}$ cDNAs were subcloned into a pCaSpeR Act-Bam injection vector (Thummel *et al.*, 1988), modified to contain unique *Xho*I and *Xba*I restriction sites adjacent to the *Bam*HI cloning site. The sequence of the two inserted cDNAs is identical, with the exception of GGA (glycine) in pCaSpeR Act/*T-r7*, versus GAA (glutamic acid) in pCaSpeR Act/*Tum-l*, at the codon for amino acid position 341. Injections were carried out as previously described (Dearolf *et al.*, 1989), except that *y w* hosts were used, and potential transformants were screened for the rescue of w^- eye pigmentation. Lethal progeny of flies injected with the pCaSpeR Act/*Tum-l* construct, and cultured at 28°C, were tested for the presence of the construct by direct sequencing of PCR products amplified from genomic DNA. The 5′ primer used was 5′ACCAATTCCGTTGCACTCGG 3′. It hybridizes to the predicted coding region (Binari and Perrimon, 1994) starting at nucleotide 912, relative to the translation start site. The 3′ primer used was 5′ACCGTT-CTCGTGCAGCTTCA 3′, which hybridizes to sequences that end at nucleotide 1352. The relevant G to A mutation occurs at nucleotide 1022. These primers yield a PCR product of 441 bp when *hop* cDNA is used as a template. Because of the presence of two introns, these primers yield a product of ~1.4 kb with *hop* genomic DNA as the template. The PCR amplification conditions used were denaturation at 94°C for 4 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and polymerization at 72°C for 1.5 min. This was followed by a final extended polymerization at 72°C for 4 min. These conditions were chosen to preferentially amplify the smaller 441 bp fragment. When the template used was control DNA from an uninjected stock, the larger band (but not the 441 bp band) was observed. For sequence determination, the 441 bp PCR product was recovered from the gel shown in Figure 6 and directly sequenced using the Promega fmol DNA sequencing system.

Acknowledgements

We thank Pamela Hanratty for her aid during the course of this work, and Norbert Perrimon for permission to use the *hop*$^+$ cDNA clone and sequence prior to publication. We acknowledge Bethany McGonnigal and Gabrielle Cramer for technical assistance with fly injections and stock maintenance. This research was supported by grants from the National Institutes of Health (HL 48823) and the Harvard Joint Center for Radiation Therapy to C.R.D. This paper is dedicated to the memory of Bill Hanratty.

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Received on December 12, 1994; revised on January 12, 1995