Cell, Vol. 84, 411–419, February 9, 1996, Copyright ©1996 by Cell Press

marelle Acts Downstream of the Drosophila HOP/JAK Kinase and Encodes a Protein Similar to the Mammalian STATs

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Summary

We have identified a putative Drosophila STAT protein named Marelle that exhibits mutant phenotypes identical to mutations in the Hopscotch/JAK kinase. We show that a reduction in the amount of *marelle* gene activity suppresses the phenotype associated with a gain-of-function mutation in *hopscotch* and enhances the phenotype associated with a weak *hopscotch* mutation. We propose that Hopscotch activates Marelle to regulate transcription of target genes such as the pair rule gene *even-skipped*. Our results demonstrate the existence of an invertebrate JAK/STAT system.

Introduction

The janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway was recently identified through studies of the transcriptional activation response to a variety of cytokines and growth factors (see reviews by Schindler and Darnell, 1995; Ihle, 1995; Ihle and Kerr, 1995; Taniguchi, 1995). Cytokines, including interferons and interleukins, are a broad group of mostly soluble factors that exert diverse effects on a number of biological processes such as immunity, hematopoiesis, and inflammation, as well as neural and embryonic development (Smith et al., 1992; Blalock, 1994). Cytokines activate receptors that do not have any obvious cytoplasmic catalytic domains. Studies on the signaling properties of these receptors have revealed the presence of two important domains. The membrane-proximal domain is responsible for the interaction with and activation of JAKs, and the more distal domain is important for the activation of the Ras pathway (Ihle and Kerr, 1995).

JAK proteins are bound to cytokine receptors through the membrane-proximal domain. Signaling is triggered when cytokine binding induces receptor dimerization. This brings the receptor-associated JAK into apposition, enabling them to transphosphorylate each other. The JAK, now activated, phosphorylate a distal tyrosine on the receptor. This receptor phosphotyrosyl residue is subsequently recognized by the SH2 domain of the STAT proteins, drawing them into the receptor complex, where they are activated through phosphorylation on a tyrosine residue by JAKs. The activated STAT proteins are now rendered competent for hetero- or homodimerization and nuclear translocation, in which they activate gene transcription (see recent reviews by Schindler and Darnell, 1995; Ihle, 1995; Ihle and Kerr, 1995; Taniguchi, 1995).

The STAT proteins are defined as a group by sequence similarities in blocks of amino acids scattered over a stretch of 700 amino acids in which is virtually the entire length of the protein (Schindler and Darnell, 1995). All known members of the STAT family have a single tyrosine residue in the region of residue 700 that becomes phosphorylated during cytoplasmic activation, and these activated proteins can bind DNA in a sequence-specific manner. Maximum sequence alignment among the family members shows strong homology within amino acid residues 600–700. This region matches the sequence of the SH2 domains of other proteins. Between amino acids 500 and 600 there is a distinct but probably meaningful similarity in the STAT proteins to the SH3 domains. The amino acids between residues 400 and 500 of STAT proteins determine the DNA-binding site specificity (Horvath et al., 1995). Some of the members of the STAT family (Stat1a, Stat3, and Stat4) have a serine residue in the region of residue 727 that can be phosphorylated (Wen et al., 1995), possibly by mitogen-activated protein kinase (MAPK), thus providing an additional level of regulation of STAT activity.

In Drosophila, a single JAK homolog, encoded by the gene *hopscotch* (*hop*), has been identified and shown to be required for normal embryonic patterning and cell proliferation (Perrimon and Mahowald, 1986; Binari and Perrimon, 1994). Embryos that develop from mothers that completely lack the wild-type *hop* gene product in their germline develop abnormally. They show a range of segmentation defects that correlate with the abnormal expression of pair rule genes such as *even-skipped* (*eve*). In addition to its function in early embryonic development, *hop* activity is required during zygotic development for cellular proliferation. Loss-of-function *hop* mutations are associated with reduced proliferation of diploid cells in the larvae, while overexpression of wild-type *hop* or a dominant gain-of-function allele, *Tumorous-lethal* ($hop^{Tum\text{-}l}$), leads to formation of melanotic tumors and hypertrophy of the larval lymph glands that represent the Drosophila hematopoietic organs (Luo et al., 1995; Harrison et al., 1995).

The discovery of HOP as a member of the JAK family of tyrosine kinases has raised the possibility that the JAK/STAT signaling pathway may exist in invertebrates. In this paper, we report the finding of an additional Drosophila gene that is associated with an embryonic mutant phenotype identical to *hop*. We have named the gene *marelle* (*mrl*), which is French for hopscotch. *mrl* encodes a Drosophila homolog of mammalian STAT. Results from genetic experiments suggest that *mrl* acts downstream of HOP in regulating transcriptional targets. This observation suggests that JAK and STAT proteins have been conserved during evolution and provide a Drosophila model to dissect the function and components of the JAK/STAT pathway.

Results

The Maternal-Effect Phenotypes of *mrl* and *hop* Are Identical

In a large screen for autosomal zygotic lethal mutations associated with specific maternal-effect lethal phenotypes, we identified a P element-induced mutation,

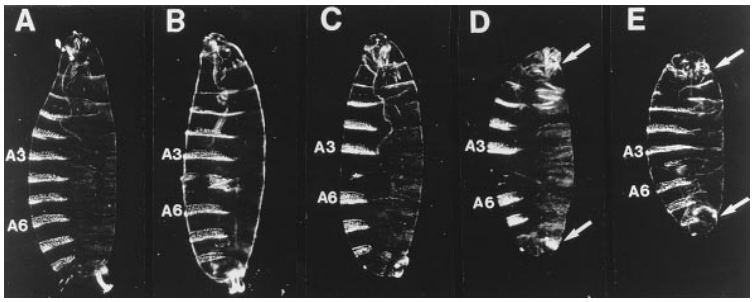


Figure 1. Abdominal Defects Associated with *mrl* and *hop* Embryos

(A) is a dark-field micrograph of a wild-type embryo showing eight abdominal dentical belts. Paternally rescued *hop* (B) and *mrl* (C) embryos are missing in abdominal segment 5, and parts of segment 4. *hop* (D) and *mrl* (E) null embryos have additional defects in the thoracic segments and the head and tail regions (arrows). A3 and A6 indicate the respective position of the segments.

l(3)06346, located at 92E2-4 (see Experimental Procedures), that exhibits a maternal-effect segmentation phenotype that is similar to the effect of loss of *hop* gene activity during oogenesis (Perrimon and Mahowald, 1986). This mutation, which we refer to as mrl^{6346}, represents a complete null mutation in a Drosophila STAT (see below); precise excision of the P element leads to homozygous viable flies, indicating that the original chromosome did not contain additional zygotic lethal mutations. *mrl* is required for zygotic viability because mutant animals homozygous for this null allele die as larvae (data not shown). The dead larvae have a normal cuticle pattern, but larval diploid imaginal tissues are reduced in size, thus implying a zygotic role for *mrl* in cellular proliferation. In addition to its zygotic function, *mrl* is required maternally for normal embryonic segmentation because *mrl* embryos derived from females lacking germline *mrl* activity (referred to as *mrl* embryos throughout the text) die with segmentation defects that resemble the phenotype of *hop* embryos (Figure 1). As is the case with *hop* embryos, the severity of the defects observed in *mrl* embryos are dependent on the paternal contribution. Both paternally rescued and unrescued *mrl* embryos show a consistent deletion of the fifth abdominal segment and the posterior midventral portion of the fourth abdominal segment. Additional defects in the thoracic segments and the head and tail region are observed in unrescued *mrl* embryos (Figure 1E). Both the zygotic and maternal phenotypes associated with a null *mrl* mutation are identical to those observed with complete loss of *hop* gene activity (Perrimon and Mahowald, 1986).

Loss of Maternal *mrl* Gene Activity Is Associated with Abnormal Pair Rule Gene Expression

mrl embryos develop abnormally and show a range of segmentation defects that correlate with the abnormal expression of pair rule genes such as *runt* (*run*) and *eve*. The most notable defect in *run* expression in *mrl* embryos consists in the almost complete loss of the fifth stripe of expression (Figure 2A3). The most prominent defect in *eve* expression in *mrl* embryos is a decrease in the third and fifth stripes (Figure 2B3). A reporter gene construct containing a 5.2 kb *eve* promoter element driving *LacZ* shows expression of *LacZ in eve* stripes 2, 3, and 7 (Goto et al., 1989). When introduced into *mrl* embryos, *eve* stripe 3 expression of this reporter gene is almost completely absent (Figure 2C3). Further, a reporter gene containing a 500 bp fragment contained within the 5.2 kb has been shown to control the expression of *eve* stripe 3 only (Small et al., 1996). When introduced into *mrl* embryos, this reporter gene is not expressed (data not shown), indicating that this 500 bp fragment identifies at least one *mrl* regulatory response element (see Discussion). All of these phenotypes are also observed in developing embryos derived from mothers that lack the wild-type *hop* gene product from their germlines (Binari and Perrimon, 1994; Figures 2A2, 2B2, and 2C2). Thus, loss of maternal *mrl* activity mimics

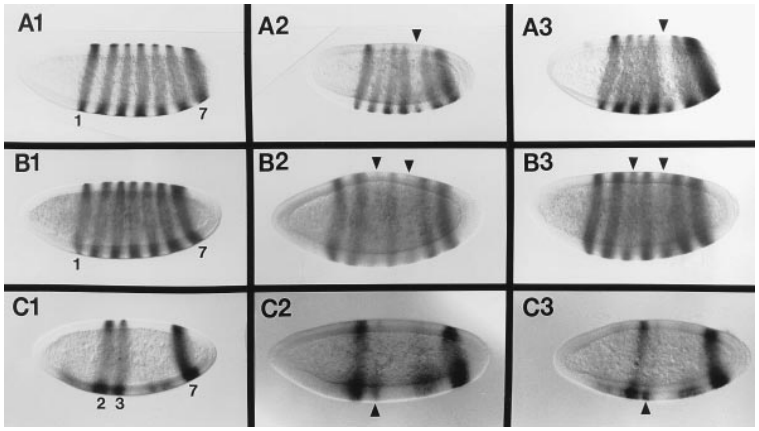


Figure 2. Pair Rule Gene Expression in Wild-Type, *hop* and *mrl* Embryos

The pattern of *run* RNA expression in wild-type (A1), *hop* (A2), and *mrl* (A3) embryos is shown. The arrowheads in (A2) and (A3) indicate the almost complete loss of the fifth stripe of *run* expression. The remaining stripes, with the exception of the first and seventh, are also variably affected in these embryos. Note that the defects are more pronounced dorsally than ventrally. *eve* RNA expression in a wild-type, *hop*, and *mrl* embryo is shown in (B1), (B2), and (B3), respectively. The arrowheads in (B2) and (B3) indicate the third and fifth stripes, which show a decrease in intensity relative to wild type. In both *hop* and *mrl* embryos, the fifth *eve* stripe is usually defective to a greater degree than the third stripe. As with *run*, the defects are more pronounced dorsally than ventrally. *lacZ expression, driven by the reporter gene construct eve* 5.2/*lacZ*, is shown in a wild-type (C1), a *hop* (C2), and a *mrl* (C3) embryo. In wild type, *eve* 5.2/*lacZ* drives *lacZ* expression in second, third, and seventh *eve* stripes. In *hop* and *mrl* embryos, *lacZ* expression corresponding to *eve* stripe three is almost completely missing (arrowhead). Embryos are oriented with anterior to the left and dorsal at the top.

Table 1. Suppression of the $hop^{Tum\text{-}1}$ Phenotype by *mrl*

Genotype	Viable	Non-*Tum*	*Tum*-
$\frac{Tum\text{-}l}{+}; \frac{+}{mrl^{6346}}$	590	555	45
$\frac{Tum\text{-}l}{+}; \frac{+}{Ly}$	37	22	15

From the same cross (see Experimental Procedures), 590 $hop^{Tum\text{-}l}/+; +/mrl^{6346}$ and 37 $hop^{Tum\text{-}l}/+;+/Ly$ females were recovered; 80% percent of the $hop^{Tum\text{-}l}/+; +/mrl^{6346}$ females and 40% of the $hop^{Tum\text{-}l}/+;l/+Ly$ females showed scorable melanotic tumors.

loss of maternal *hop* gene activity, suggesting that *mrl* and *hop* operate in the same developmental pathway.

Dosage Interactions between *mrl* and *hop*

To determine whether *hop* and *mrl* genetically interact, we tested whether a reduction in the amount of maternal *mrl* gene activity could enhance the maternal effect associated with a partial loss of function *hop* mutation. Embryos that are derived from mothers that carry germline clones of the hop^{msv1} hypomorphic allele show weak segmentation defects (Perrimon and Mahowald, 1986; Figure 3A), and many of them hatched. However, when these embryos are derived from females that also carry a single copy of *mrl*, they exhibit segmentation defects that are similar to embryos that are derived from females lacking all maternal *hop* activity (Figures 3B and 3C; Perrimon and Mahowald, 1986), and none of them hatched. This result strongly suggests that *hop and mrl* act in concert to regulate embryonic segmentation.

mrl Is Epistatic to *hop*

We analyzed whether MRL operates upstream or downstream of HOP by testing whether the effect of a hyperactive *hop* allele could be negated by a reduction in the amount of *mrl* gene activity. If *mrl* is required to transduce the HOP signal, then a reduction in *mrl* gene activity should suppress a *hop* gain-of-function phenotype. We utilized the dominant temperature-sensitive *hop* allele, $hop^{Tum\text{-}l}$, for this experiment. When grown above 25°C, flies heterozygous for $hop^{Tum\text{-}l}$ have reduced viability, and the emerging adults develop melanotic tumors (Corwin and Hanratty, 1976; Table 1).

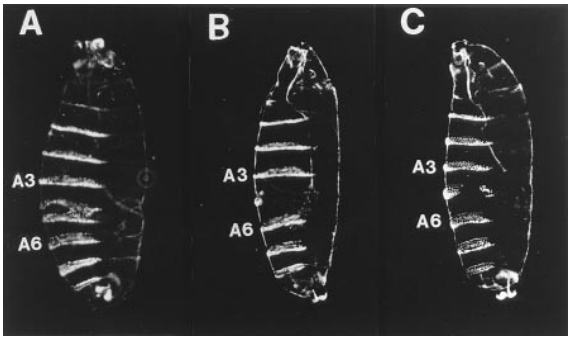


Figure 3. *mrl* Enhances the *hop* Phenotype

The dark-field cuticle pattern of an embryo derived from females that carry hop^{msv1} germline clones (A) is compared with embryos derived from females heterozygous for mrl^{6346} that carried hop^{msv1} homozygous germ line clones (B and C). More than 200 embryos from each case have been compared in this experiment. Only paternally rescued embryos are shown here. Unrescued embryos have additional defects in head and tail regions, and *mrl* also enhances these *hop* phenotypes (data not shown).

The viability and formation of melanotic tumors at 29°C was compared in females heterozygous for $hop^{Tum\text{-}l}$ and mrl^{6346} with females heterozygous only for $hop^{Tum\text{-}l}$ (see Experimental Procedures). We observed an improved survival rate and a significant reduction in the formation of melanotic tumors by removing a single copy of *mrl* in $hop^{Tum\text{-}l}$ heterozygous females (Table 1). This result strongly suggests that *mrl* encodes a component of the HOP signal transduction pathway and functions downstream of the HOP tyrosine kinase.

Molecular Cloning of the *mrl* Gene

To identify the gene product encoded by *mrl*, we cloned the genomic sequences flanking the P element insertion associated with mrl^{6346} following plasmid rescue. Subfragments of the rescued genomic DNA were then used to screen various cDNA and genomic libraries (for details see Experimental Procedures; Figure 4). The largest cDNA recovered was a full-length 4.0 kb clone and encodes a conceptual protein of 761 amino acids (Figure 4).

The following lines of evidence argue that the cDNA we isolated (Figure 4B) corresponds to *mrl* function. The phenotype and lethality associated with mrl^{6346} can be reverted to wild type by the mobilization of the P element insertion (see Experimental Procedures). The P element is inserted in an intron that separates the promoter and first exon from the main body of the coding sequence (see Experimental Procedures and legend to Figure 4A). Further, the insertion of the P element at this site leads to the absence of detectable levels of *mrl* transcript in homozygous mrl^{6346} embryos derived from females with germline clones (Figure 5D; see Figure 6), lane 0–1.5 *mrl*. This indicates that the 15 kb P element sequence interferes with the proper expression of the transcription unit represented by the cDNA we isolated. One *mrl* lethal mutation (mrl^{42}) generated by the imprecise excision of the mrl^{6346} transposon was associated with a deletion of the genomic region that contains the transcription initiation site and 5′ untranslated sequence of *mrl* cDNA (see Figure 4A). Finally, RNA transcribed in vitro from the cDNA insert provided rescuing activity when injected into mrl^{6346} embryos derived from germline clones (Figures 5E and 5F).

mrl Encodes a Protein Homologous to the Mammalian STAT Transcription Factors

The amino acid sequence derived from the *mrl* cDNA we isolated is shown in Figure 4B. Computer-assisted homology searches in the GenBank and EMBL databases revealed that *mrl* shares a distinct homology to the human STAT5 and STAT6 proteins (Hou et al., 1994, 1995a). The overall identity between *mrl* and STAT5 and *mrl* and STAT6 is 37.0 and 34.7%, respectively. Like the STAT proteins, *mrl* contains a src homology 2 (SH2)-like domain and a DNA-binding domain (Figure 4C). A highly conserved motif, GTFLLRFSDES, is found in the

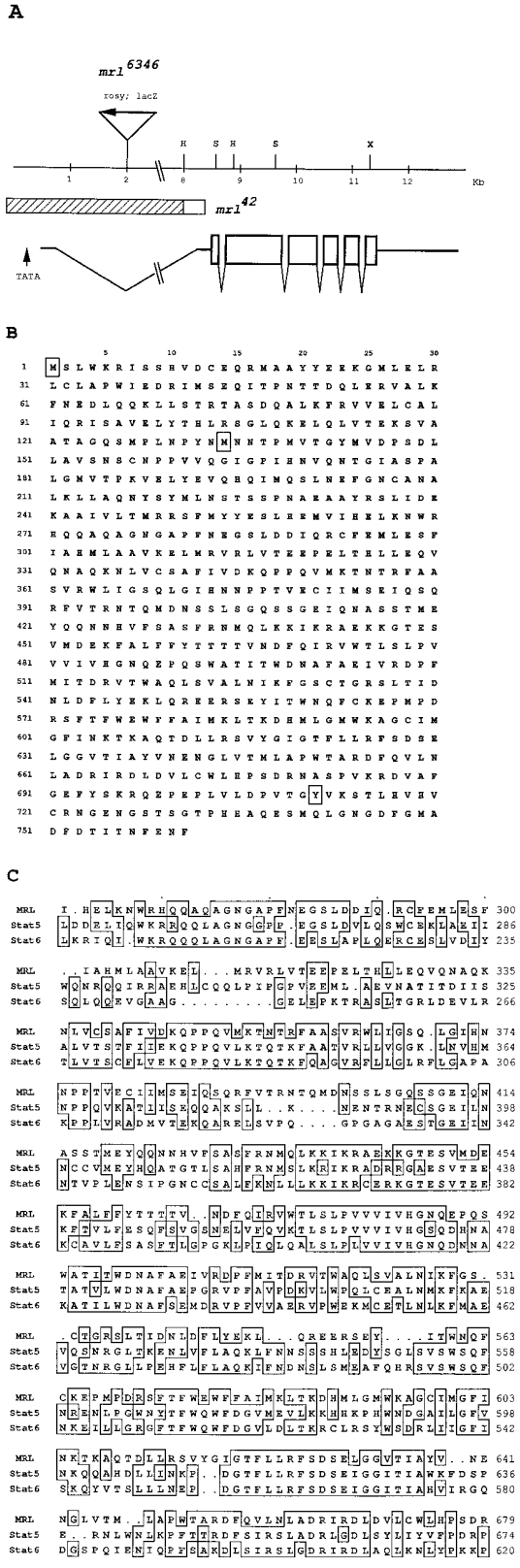


Figure 4. Intron–Exon Structure of the *mrl* Gene

(A) Comparison of a 4.0 kb *mrl* cDNA and genomic DNA from the locus reveals that the *mrl* transcript is made up of seven exons. The first, a 200 bp exon consisting of 5′ untranslated DNA, is separated from the remaining exons by approximately 7 kb. A presumptive transcription start site or TATA box is found in the genomic DNA about 180 bp 5′ to the start of the cDNA. The insertion site of the *mrl*^{6346} transposon is found 1.3 kb 3′ to the first *mrl* exon. The putative *mrl* open reading frame, represented in this figure by open boxes, spans the next six exons. The extent of DNA deleted in the *mrl*^{42} lethal mutant was analyzed by genomic DNA blotting and is indicated under the diagram of the genomic DNA; the hatched bar indicates sequence missing in the allele, and the open bar, indicates uncertainty as to the end of the deletion. H, HindIII; S, SalI; X, XbaI.

(B) Putative amino acid sequence of *mrl*. The translation start site shown was chosen because it is found at the beginning of a long open reading frame with good Drosophila codon usage. It is not, however, preceded by a good translation initiation consensus sequence (GAGC instead of the consensus [C/A]AA[C/A] [Cavener, 1987]. The highlighted methionine 134 amino acids later, in contrast, is preceded by a perfect translation initiation sequence (CAAC) and thus must formally be considered as a potential translation start site. The first 134 amino acids of the longer open reading frame show no homology to other STATs. The longer, 761 amino acid (86 kDa predicted molecular mass) protein would, however, be more comparable in length to the other STATs than the 629 amino acid, 71 kDa protein corresponding to the second potential start site. The putative phosphotyrosine is indicated.

(C) Comparison of the Predicted MRL Protein to Human STAT5 and STAT6 in the Region of Their Most Significant Homology

MRL shows 45% amino acid identity with human STAT5 (Hou et al., 1995a), and 41% identity with human STAT6 (Hou et al., 1994) in the region shown.

potential SH2 domain of MRL, and a highly conserved motif, TLSLPVVVIVHG, is found in the potential DNA-binding domain of MRL. Although MRL and the some STAT proteins share homologies throughout, they also exhibit distinct differences. The SH3-like domain found in some STAT proteins is less clear in MRL, and the putative MAPK phosphorylation site present in some STAT proteins is missing in the MRL sequence. MRL contains a putative tyrosine phosphorylation site at position 711 that is in the same relative position as in the STAT5 protein (VXGYVK; Hou et al., 1995a).

Expression of *mrl* during Embryogenesis

Developmental Northern blot analysis using the *mrl* cDNA as a probe detects three transcripts of 4.0, 3.5, and 3.0 kb in size. The 4.0 kb species is the most abundant and is expressed throughout embryonic, larval, pupal, and adult stages. The other two smaller transcripts, at 3.5 and 3.0 kb, are detected during larval stages. The 4.0 kb cDNA we isolated most likely represents a full-length message, since it is similar in size to the major 4.0 kb transcript observed on Northern blots. Further, when injected into embryos derived from females with *mrl* germline clones, RNA prepared from this cDNA is capable of rescuing the *mrl* maternal-effect segmentation defects (Figures 5E and 5F). To examine the pattern of *mrl* expression during embryogenesis, we hybridized the whole-mount embryos with digoxygenin-labeled *mrl* cDNA probe (see Experimental Procedures). High and uniformly distributed levels of MRL RNAs were detected in early syncytial and cellularizing embryos, a pattern very similar to the one observed for *hop* transcripts (Figures 5A and 5B; R. Binari and N.P., unpublished data). During germband extension, *mrl* transcripts

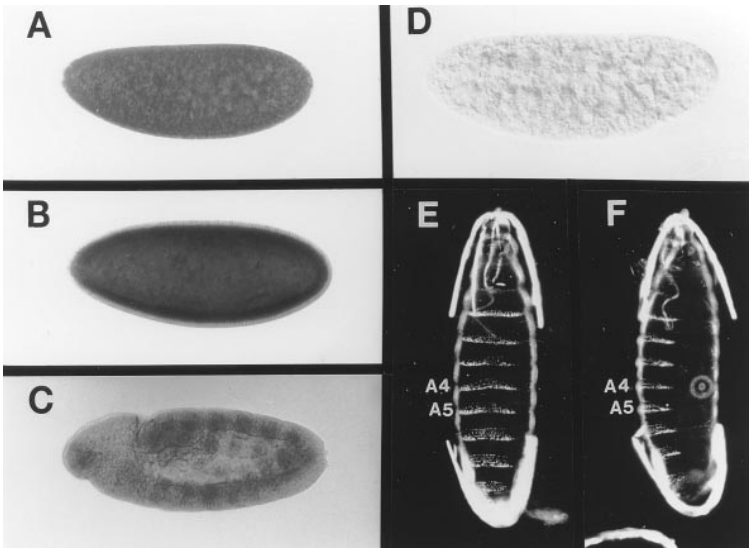


Figure 5. *mrl* Expression during Early Development

The embryonic expression pattern of *mrl* has been analyzed by whole-mount in situ hybridization with a *mrl* antisense DNA probe. *mrl* RNA is expressed uniformly in preblastoderm (A) and in early blastoderm stage (B) embryos. During germband extension, *mrl* is expressed predominantly in 14 mesodermal stripes (C). *mrl* expression is completely abolished in embryos derived from females with mrl^{6346} germline clones (D). (E) and (F) are two examples of the cuticle phenotypes of mrl^{6346} embryos derived from germline clones injected with RNA transcribed from 4.0 kb *mrl* cDNA. Note the rescue of the abdominal denticle bands A4 and A5 that are missing in non rescued mrl^{6346} embryos. Of injected embryos, 150 developed scorable cuticle structures, and 50% of them showed significant rescue of the abdominal denticle bands A4 and A5.

were detected in a striped pattern within every segment (Figure 5C).

Discussion

We have identified a novel Drosophila STAT protein, MRL, which shares homology with the family of STAT proteins. We find that mutations in *mrl* have a maternal-effect phenotype similar to mutations in the previously isolated Drosophila JAK hop. Specific genetic interactions between mutations in the two genes suggest that *mrl* functions downstream of the HOP tyrosine kinase.

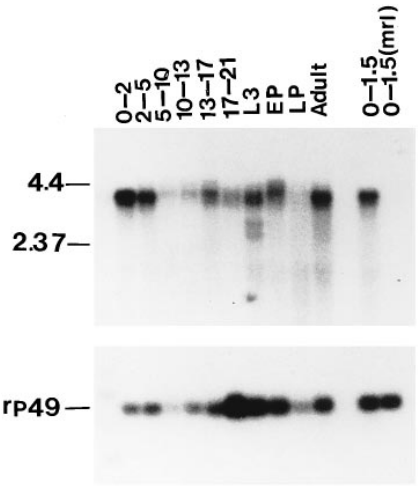


Figure 6. Developmental Northern Blot Analysis of *mrl* Expression

Poly(A)$^+$RNA, prepared from various developmental stages, was loaded in each lane, electrophoresed, transferred to nitrocellulose, and probed with a ^{32}P-labeled 4.0 kb *mrl* cDNA. Lanes are marked according to the specific developmental stage: numbers during embryonic stages refer to hours of development after fertilization; L3 refers to third instar larvae; early pupae (EP) are 0–24 hr after pupation; late pupae (LP) are 96–120 hr after pupation; adult RNA is from a mixed population of both males and females; and 0–1.5 (*mrl*) is RNA from 0–1.5 hr mrl^{6346} embryos, which is compared with RNA from wild-type embryos of the same stage (0–1.5). The 4.0 kb transcript is observed throughout development and is completely missing in 0–1.5 (*mrl*) embryos. Two short transcripts, of approximately 3.5 kb and 3.0 kb, appear only during the larval stage. The lower panel shows the same Northern blot hybridized with a ribosomal protein gene 49 sequence (O'Connell and Rosbash, 1984).

mrl Encodes a Novel STAT Protein

The protein encoded by the *mrl* gene shares 37% identity with the human STAT5 (Hou et al., 1995a). The overall structural similarity between the two proteins is remarkable in that both contain a DNA-binding domain, an SH2 domain, and a putative tyrosine phosphorylation site. Examination of segmentation gene expression in embryos devoid of maternal *mrl* expression indicates that *mrl* is involved in the regulation of pair rule genes such as *eve* and *run*. We have identified a 500 bp minimal promoter fragment in the *eve* promoter that responds to *mrl* activity. In wild-type embryos, this 500 bp fragment drives expression of *lacZ* RNA in *eve* stripe 3; however, in the absence of maternal *mrl* activity, *lacZ* expression from this promoter is not observed. Thus, this region must contain at least one *mrl* regulatory response element. By examining the sequence of the 500 bp DNA fragment that was made available to us by Small et al. (1996), we found two sequences in this 500 bp *eve* stripe 3 enhancer, TTCCCCGAA and TTCCGCGAA, that closely matches the mammalian STAT-binding site (Figures 7B and 7C). Further analysis should reveal whether activated MRL protein binds to these sites to regulate *eve* expression.

Function of the Drosophila JAK/STAT Pathway in Pair Rule Gene Regulation

Studies on the mechanism by that specific pair rule genes become expressed in stripes have revealed that different combinations of gap gene proteins act on individual stripe-specific promoter elements (Pankratz et al., 1990; Stanojevic et al., 1991). For example, the expression of *eve* stripe 2 is the result of a combination of overlapping activators and repressors. In the case of

eve stripe 2, Bicoid (BCD) and Hunchback (HB) proteins mediate activation, whereas Krüppel (KR) and Giant (GT) determine the borders of the stripe through repression (Small et al., 1991, 1992). In the case of *eve* stripe 3, the anterior and posterior borders are defined through repression by HB and knirps (KNI), respectively. It has been speculated that perhaps one or more products of the gap genes may correspond to activators (Stanojevic et al., 1991; Small et al., 1996). However, these have not yet been identified. As previously suggested by Binari and Perrimon (1994), HOP may be a component of this activation mechanism. Thus, we propose that MRL, when activated by HOP, may correspond to the positive transcription factor that activates *eve* stripe 3 expression. If the Drosophila JAK/STAT pathway is regulated by the same mechanism as the vertebrate pathway, we expect that a transmembrane receptor will regulate HOP/MRL activity. Identification of additional mutants with similar effects on *eve* regulation may lead to the identification of such a receptor.

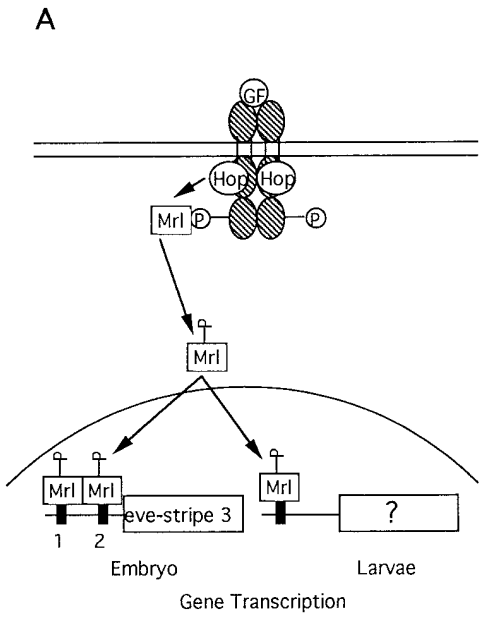
A striking effect of loss of HOP/MRL activity is the stripe-specific effect observed on pair rule gene expression. This specificity does not necessarily imply that the mechanism of activation of this pathway is localized. Perhaps the HOP/MRL system is constitutively activated in early embryos, and the specificity of the response simply reflects the structure of the regulatory regions in the promoter of the downstream targets. Genes that contain multiple MRL-binding sites in their promoters may become activated at higher levels than others. Such a regulatory system in the context of *eve* stripe 3 may compensate for the lack of positive input from the gap genes.

Function of the Drosophila JAK/STAT Pathway in Cell Proliferation

A dominant gain-of function *hop* allele, $hop^{Tum\text{-}l}$, leads to formation of melanotic tumors and hypertrophy of the larval lymph glands, the Drosophila hematopoietic organs (Luo et al., 1995; Harrison et al., 1995). The $hop^{Tum\text{-}l}$ allele encodes a hyperactive HOP kinase (Harrison et al., 1995). A loss-of-function mutation of *mrl* suppresses the phenotype of $hop^{Tum\text{-}l}$, suggesting that *mrl* is involved in the HOP pathway that regulates cell proliferation in larval blood cells. During blood cell formation, HOP/MRL activity may be regulated by a growth factor receptor, perhaps a cytokine receptor like the vertebrate erythropoeitin receptor or the epidermal growth factor (EGF) receptor (see Luo et al., 1995; Harrison et al., 1995). Identification of a transmembrane receptor that activates this pathway will be necessary to understand the function of the HOP/MRL pathway in cell proliferation and tumorigenesis.

Model of Activation

In Figure 7A, we show a model by which the HOP/MRL pathway regulates gene expression. This model integrates the results presented in this paper, as well as the current view of the mammalian JAK/STAT pathway (Schindler and Darnell, 1995). We propose that, like other mammalian JAK/STAT pathways, HOP is associated with a transmembrane receptor lacking a kinase domain.



B

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1   GGATCCTCGA AATCGAGAGC GACCTCGCTG CATTAGAAAA CTAGATCAGT
51  TTTTTATTTT GGCCGACCGA TTTTTGTGCC CGGTGCTCTC TTTACGGTTT
101 ATGGCCGCGT TCCCATTTCC CAGCTTCTTT GTTCCGGGCT CAGAAATCTG
151 TATGGAATTA TGGTATATGC AGATTTTTAT GGGTCCCGGC GATCCGGTTC
201 GCGGAATGGG AGTGTCCTGC CGCGAGAGGT CCTCGCCGGC GATCCTTGTC
251 GCCCGTATTA GGAAAGAGAT CACGTTTTTT GTTCCCATTG TGCGCTTTTT
301 TGCTGCGCTA GTTTTTTTCC CGGAACCCAG CGAACTGCTC TAATTTTTTA
351 ATTCTTCACG GCTTTTCATT GGGCTCCTGG AAAAACGCGG ACAAGGTTAT
401 AACGCTCTAC TTACCTGCAA TTGTGGCCAT AACTCGCACT CTCTCGTTTT
451 TAAGATCCGT TTGTTTGTGT TTGTTTTGTCC GCGATGGCAT TCACGTTTTT
501 ACGAGCTCGT TCC
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C

eve 1	TTCGCGGAA
eve 2	TTCCCCGAA
β-casein	TTCTAGGAA
Consensus	TTCXXXGAA

Figure 7. Model of HOP/MRL Function

(A) Model of HOP/MRL signal transduction. See text for details. Boxes 1 and 2 refer to the two potential STAT-binding sites on the *eve* stripe 3 enhancer. (B) The 500 bp sequence of the *eve* stripe 3 enhancer (Small et. al., 1996) underlined are two potential STAT binding sites. (C) Comparison of the two potential STAT- binding sites on the *eve* stripe 3 enhancer with MGF-STAT5 binding site from the β-casein gene promoter (Wakao et al., 1992).

Binding of a ligand to this unknown receptor will promote receptor dimerization, bringing the receptor-associated HOP molecules into apposition. As a result of this apposition, HOP kinases will transphosphorylate each other, as well as phosphorylate a tyrosine on the receptor. Subsequently, these receptor phosphotyrosyl residues can be recognized by the SH2 domain of MRL molecules, drawing them into the receptor complex, where they become tyrosine phosphorylated by HOP. The phosphorylated and thus activated MRL protein can subsequently enter the nucleus and activate gene transcription. Loss-of-function *mrl* mutations both enhance the embryonic phenotypes of the hypomorphic hop^{msv1}

allele and suppress the phenotypes of the gain-of-function *hop*$^{Tum\text{-}l}$ allele. These results suggest that the same pathway functions during embryonic and larval development. In the embryo, activated MRL may bind to the promoters of pair rule genes, for example, the *eve* stripe 3 enhancer, and activate their expression. In larvae, activated MRL may bind to the promoters of genes involved in cellular proliferation. Identification of transmembrane receptor(s) that activates this pathway, as well as downstream targets that respond to this pathway, will be necessary to substantiate this model.

HOP and MRL Are Most Similar to JAK2/STAT5

Among all STATs isolated to date, MRL shows the highest homology to the mammalian STAT5. The potential MRL-binding sites TTCCCCGAA and TTCCGCGAA in the *eve* stripe 3 enhancer best match the TTCXXXGAA consensus for STAT5 binding (Pallard et al., 1995). Taken together, these observations suggest that MRL is the Drosophila counterpart of the mammalian STAT5. STAT5 was originally identified as a transcription factor activated by prolactin in the lactating mammary gland (Wakao et al., 1994). STAT5 was shown to be strictly required for the induction of transcription of milk caseins. However, it has been reported to be more widely expressed, being present in the spleen and thymus, as well as in other tissues (Wakao et al., 1994). STAT5 is activated by the JAK2 tyrosine kinase in vitro (Gouilleux et al., 1994). JAK2 is a kinase that is associated with and activated by the receptors for prolactin, erythropoietin, growth hormone, granulocyte colony-stimulating factor, interleukin-5, granulocyte-macrophage colony-stimulating factor, interleukin-3, interleukin-6, and interferon (Argetsinger et al., 1993; Muller et al., 1993; Silvennoinen et al., 1993; Watling et al., 1993; Witthuhn et al., 1993; Ihle et al., 1994; Narazaki et al., 1994; Quelle et al., 1994). JAK2 activation by these receptors in turn results in the activation of STAT5 (Gouilleux et al., 1995). Among the JAK family members, HOP protein shows the highest degree of identity with JAK2 (27%; Binari and Perrimon, 1994), suggesting that HOP may be the Drosophila counterpart of the mammalian JAK2. An interesting aspect of these evolutionary considerations is their suggestion that the Drosophila transmembrane receptor(s) that activates the HOP/MRL pathway may be a member of the receptor family that regulates JAK2.

Concluding Remarks

Results presented in this paper demonstrate the existence of at least one Drosophila JAK/STAT pathway. This pathway is involved in at least two developmental pathways, regulation of pair rule gene expression and cell proliferation. It is possible that additional JAK and STAT genes exist in Drosophila, and it will be important to determine whether this is the case. The availability of a genetic system to study this pathway provides a useful model to dissect the function and signaling mechanism of a JAK/STAT pathway further.

Experimental Procedures

Drosophila Stocks

mrl^{6346} was identified in a large screen for autosomal zygotic lethal mutations associated with specific maternal-effect phenotypes. Details of this screen will be published elsewhere (N.P. et al., unpublished data). *mrl*^{6346} originated from the collection of third chromosome lethal mutations described by Spradling et al. (1995).

To determine whether the insertion of the enhancer trap P element was responsible for the mutant phenotype, we mobilized the P element associated with the *mrl*^{6346} mutation using the *y w; Δ2–3, Sb/TM6* strain (Robertson et al., 1988). Of the excision lines, 79 were wild-type, and one *mrl*^{42} was associated with zygotic lethality.

Three *hop* alleles were used in this study: *C111*, a null allele; *msv1*, a weak allele (Perrimon and Mahowald, 1986); and *Tum-l*, a dominant temperature-sensitive allele (Corwin and Hanratty, 1976).

Flies were raised on standard Drosophila media at 25°C, unless indicated. Chromosomes and mutations that are not described in the text can be found in Lindsley and Zimm (1990).

Germline Clones

Females carrying germline clones of *mrl* were generated using the FLP-DFS technique (Chou et al., 1993; Hou et al., 1995b; T.B. Chou and N.P., unpublished data). Virgin females of the genotype *TM3, Sb/FRT*^{82B}*mrl* were mated with males of the genotype *y w FLP*^{22}*; FRT*^{82B} *P [ovo*^{D1}*]*^{a31n9}*/TM3, Sb*. The resulting progeny were heat shocked at 37°C for 2 hr at the larval stages, and *y w FLP*^{22}*/+; FRT*^{82B}*mrl/FRT*^{82B} *P[ovo*^{D1}*]*^{a31n9} females carrying *mrl* homozygous germline clones were selected. Embryos derived from these females are referred to as *mrl* embryos. To determine the phenotype of rescued (*mrl*/+) versus unrescued (*mrl*/*mrl*) *mrl* embryos, we crossed females with *mrl* germline clones with either +/+ or +/*mrl*, and the embryos scored for embryonic defects.

Genetic Interaction between *hop* and *mrl*

To test for interaction between *hop* and *mrl*, we compared the cuticle phenotype of eggs derived from females heterozygous for *mrl*^{6346} that carried *hop*^{msv1} homozygous germline clones with eggs derived from females that carry *hop*^{msv1} germline clones. To generate such females, we crossed progeny from *hop*^{msv1} *FRT*^{101}*/FM7; mrl*^{6346}*/TM3, Sb* females with *ovo*^{D1}*FRT*$^{101}{}_{/Y}$; FLP^{38}/FLP^{38} males for 2 hr at 37°C during larval stages (see Chou and Perrimon, 1992). Females of genotypes *hop*^{msv1} *FRT*^{101}*/ovo*^{D1} *FRT*^{101}*; +/mrl*^{6346} and *hop*^{msv1} *FRT*^{101}*/ovo*^{D1} *FRT*^{101}*; and +/TM3, Sb* were crossed to *mrl*^{6346}*/TM3, Sb* males and raised at 25°C.

To analyze the genetic epistasis between *hop* and *mrl*,+ we generated females of genotypes *hop*$^{Tum\text{-}l}$*/+; +/mrl*^{6346} and *hop*$^{Tum\text{-}l}$*/+; +/Ly* from the cross of *hop*$^{Tum\text{-}l}$*/FM7* females with +/*Y; mrl*^{6346}*/Ly* and scored them at 29°C for viability and the presence of melanotic tumors.

In Situ Hybridization

In situ hybridization to whole-mount embryos using digoxigenin-labeled antisense DNA probes were performed as described (Hou et al., 1995b). Probes were prepared from plasmids containing the following sequences: *eve* cDNA (p572–B7; 0.9 kb of *eve* coding sequence cloned into pGEM1); *run* cDNA (pED5′; the entire *run* coding sequence cloned into pBluescript (SK^{+}); and *lacZ*–coding region (a 2.4 kb BamHI–XbaI fragment from pC4Gal; Thummel et al., 1988). To test whether the sequences corresponding to our putative *mrl* cDNA were transcribed in *mrl*^{6346} embryos or not, we collected *mrl*^{6346} embryos and used them for in situ hybridization with an antisense DNA probe derived from the *mrl* cDNA.

Isolation of Genomic and cDNA Clones

Sequencing both ends of the XbaI rescued fragment revealed an open reading frame at the distal side of the P element insertion. There was no open reading frame just adjacent to the P element insertion. Genomic DNAs covering the *mrl* locus were isolated from a Drosophila genomic library made in bacteriophage EMBL3 (Blackman et al., 1987). To isolate the *mrl* cDNA, we screened a 0–4 hr

embryonic cDNA library (Brown and Kafatos, 1988) with random-primed probes (Feinberg and Vogelstein, 1983) generated from genomic DNA fragments from the *mrl* locus. Two full-length 4.0 kb clones and six shorter cDNA clones, corresponding to one open reading frame, were isolated. The longest 4.0 kb clone was selected for further analysis.

DNA Sequencing

DNA sequencing was carried out using a dideoxy chain termination protocol (Sanger et al., 1977; Del Sal et al., 1989) and Sequenase (United States Biochemical Corporation). The entire sequence of the cDNA and the corresponding genomic sequence were sequenced by use of consecutive oligonucleotide primers synthesized to extend the sequences. The positions of the intron–exon boundaries were determined by comparison of the sequence of genomic DNA to that of the cDNA. DNA sequence analysis was carried out with the Wisconsin Genetics Computer Group sequence analysis programs (Devereux et al., 1984). GenBank and EMBL databases were searched by use of the TFASTA, BLASTP, and WORDSEARCH programs. Alignments were generated using the BESTFIT program.

RNA Analysis

Northern blotting and probe preparation were carried out according to standard methods (Sambrook et al., 1989). Approximately 5 μg of poly(A)$^+$ RNA per lane was fractionated on a 1% formaldehyde agarose gel and transferred to nitrocellulose.

RNA Microinjection

RNA was produced from plasmid templates containing an SP6 promoter as described (Krieg and Melton, 1987), using the cap analog GpppG (Strategene). Transcribed RNA was resuspended in DEPC-treated water at a concentration of 0.2 μg/μl. *mrl*^{6346} embryos were collected from females with germline clones and microinjected as previously described (Anderson and Nüsslein-Volhard, 1984). Embryos were allowed to complete development for 3 days at 18°C prior to preparation, and scoring of cuticles was as described (Wieschaus and Nüsslein-Volhard, 1986).

Acknowledgments

We thank K. Matthews for providing us with the *mrl* P element induced–mutation; R. Yan, S. Small, C. Dearolf, C. Desplan, and J. Darnell for communicating data before publication; M. Gilman for useful discussions; and L. Perkins for comments on the manuscript. We especially thank S. Small for providing data regarding the sequence of the *eve* stripe 3 enhancer. This work was supported by the Howard Hughes Medical Institute, where N. P. is an Associate Investigator.

Received November 22, 1995; revised January 4, 1996.

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GenBank Accession Number

The accession number for the sequence reported in this paper is U40070.