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Microarray analyses reveal distinct roles for Rel proteins in the *Drosophila* immune response

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Abstract

The NF- κ B group of transcription factors play an important role in mediating immune responses in organisms as diverse as insects and mammals. The fruit fly *Drosophila melanogaster* express three closely related NF- κ B-like transcription factors: Dorsal, Dif, and Relish. To study their roles *in vivo*, we used microarrays to determine the effect of null mutations in individual Rel transcription factors on larval immune gene expression. Of the 188 genes that were significantly up-regulated in wildtype larvae upon bacterial challenge, overlapping but distinct groups of genes were affected in the Rel mutants. We also ectopically expressed Dorsal or Dif and used cDNA microarrays to determine the genes that were up-regulated in the presence of these transcription factors. This expression was sufficient to drive expression of some immune genes, suggesting redundancy in the regulation of these genes. Combining this data, we also identified novel genes that may be specific targets of Dif.

Keywords

Dorsal; Dif; Relish; NF- κ B; Antimicrobial Peptide; Toll; Target genes

INTRODUCTION

Innate host defenses are found in all multicellular organisms. In metazoans as diverse as mammals and insects, the NF- κ B class of transcription factors plays a conserved role in mediating innate immune responses. Mammals express five proteins of the NF- κ B family- p50, p52, p65, p100 and p105. These proteins dimerize to produce a large number of transcriptional elements that can regulate genes important for a variety of cellular processes, including immune and inflammatory responses, growth and development, and cell death. Misregulation of NF- κ B has been linked to cancer, autoimmune diseases, and viral proliferation. [1-3]. Because of significant conservation in the signaling pathways responsible for NF- κ B activation as well as the structure of the proteins themselves, the fruit fly *Drosophila melanogaster* has been an attractive model to study these transcription factors.

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The fruit fly expresses three Rel/NF- κ B proteins: Dorsal, Dif, and Relish. These proteins share a Rel homology domain that dimerizes to bind DNA and initiate transcription. Two signal transduction pathways, Toll and imd, are involved in activating these three Rel proteins in response to infection [4,5]. Recognition of microbial products by upstream pattern recognition receptors causes the activation of these pathways in immune tissues of *Drosophila*. Toll activation causes phosphorylation and degradation of an inhibitor of κ B (I κ B), Cactus. Cactus sequesters Dorsal and Dif in the cytosol, and its degradation releases them to translocate to the nucleus and initiate transcription of genes [6,7]. The imd pathway regulates Relish activation in a similar way: signal-dependent cleavage of the inhibitory ankyrin repeat domain of Relish leads to release of the Rel domain responsible for transcription [8-10]. During an immune response, differential Toll and imd pathway activation of the three NF- κ B proteins is believed to cause different transcriptional outcomes [11]. *In vitro* evidence demonstrates heterodimerization and homodimerization of the three Rel proteins can create transcription factors with different target specificities [12]. SELEX assays have identified that Dorsal and Dif/Relish have greater affinity for different 9–12 base pair sequences [13]. Due to the presence of cis-acting transcription factor binding sites with affinity for different dimer combinations, it is predicted that different sets of genes may thus be controlled by the three Rel proteins. However, few attempts have been made to explore this question *in vivo*.

The antimicrobial peptides (AMPs) in the fat body are an important class of proteins differentially regulated in this way [14]. The humoral immune response is characterized by the induction of these AMPs in the fat body of the fly in response to infection. These peptides have antimicrobial activity and help subdue the infection. The fruit fly also mounts cellular responses, such as phagocytosis or encapsulation of microbes by circulating hemocytes, and proteolytic cascades that result in melanization toxic to infectious agents at wound sites [15-17]. Rel proteins have been well characterized for their central role in mediating the humoral response, but they may also induce genes involved with these other aspects of the *Drosophila* immune response. However, studies of Rel transcription factors have usually focused on their role in regulating expression of the antimicrobial peptide (AMP) genes.

Flies lacking Dorsal do not appear to be compromised in their ability to induce any of the known AMPs [18]. Whereas *Dif* mutant flies have greatly reduced abilities to induce *Drosomycin* and *Defensin* [19,20] and *relish* mutant flies are incapable of inducing *Diptericin* and *Cecropin* during infection [8]. On the other hand, expression of Rel proteins in tissue culture cells reveals that Dif and Relish together form the most potent activator of *Drosomycin*, *Defensin*, and *Attacin* [12]. Expression of Dif alone can induce *Cecropin* and *Diptericin* expression, while expression of Dorsal or Relish alone, does not result in induction of any of these AMPs. While these studies hint at considerable regulatory complexity resulting from the heterodimerization of Rel proteins, they have focused solely on the induction of AMP genes. The broader effects of Rel mutations in regulating global gene expression during an immune response are not known, so we sought to explore this question *in vivo*.

Using a microarray approach, we examine the effect on immune gene expression when the Rel proteins are absent due to mutation or when they are ectopically expressed. The Rel proteins that are necessary or sufficient for the expression of these immune genes could therefore be determined, by analyzing gene expression in mutant or Rel expressing flies respectively. Using Affymetrix *Drosophila* GeneChips, we identified 188 genes that were induced upon Gram[−] bacterial infection in wildtype OregonR (OR) larvae. Among these genes, overlapping but different subsets of these genes failed to induce to wildtype levels in *dorsal*, *Dif*, and *relish* mutant larvae. A substantial percentage of these affected genes were involved with mediating the flies' immune responses, with most of the known AMPs affected by *Relish* or *Dif* mutations. Redundancy between Rel proteins may account for failure to see the effects of single mutants, so we also looked at global gene expression resulting from ectopic expression of Dorsal or Dif.

A number of genes important for immunity were induced in both cases, including some that were not identified from the loss-of-function experiments. A comparison of these datasets enabled a global characterization of the role of the Rel proteins in mediating gene expression in an immune response. It also enabled the identification of putative target genes. To date, understanding the Toll pathway has been limited because the only known target gene has been *Drosomycin*. Other groups have identified Toll dependent genes from microarray studies, but they have not been validated or used as target genes for the Toll pathway [21]. Here we report the identification of several novel Toll target genes that are induced more rapidly, and are more specific indicators of Toll pathway activation.

RESULTS AND DISCUSSION

Rel proteins have overlapping but distinct functions

For an unbiased genomic level perspective on the roles that Dorsal, Dif, and Relish play during an immune response, we used Affymetrix oligonucleotide microarrays to study gene expression in flies' mutant for these transcription factors. Third instar larvae were accurately staged and injected with *E. coli*. Two hours later, RNA was extracted, labeled, and hybridized to the microarrays. Along with the wildtype larvae, we injected and studied gene expression in Rel mutant larvae *dorsal*¹, *Dif*¹, and *relish*^{E20}. Of the 13,500 transcripts represented on the microarray, 188 genes were significantly induced by infection in wildtype flies. For these genes, the effect of a given Rel mutation was calculated as an expression ratio, comparing expression in the Rel mutant to wildtype levels (Supplementary Table 1). A selection of genes induced or repressed in the Rel mutants is presented (Table 1). Other groups have performed similar microarray experiments to study gene expression during infection in adults and *Drosophila* S2 cells [21-23] but this is the first time a comparison of the effect of specific Rel mutations on gene expression has been examined.

All studies to date have explored the role of Rel proteins on the induction of AMP genes. We found a number of AMP genes that are poorly expressed in *relish* mutants including *Diptericin*, *Cecropin*, *Defensin*, *Attacin*, and *Drosomycin*, confirming its key role in the humoral immune response. Other transcripts affected in Relish mutants that are categorized as being important for the defense response by Gene Ontology (GO:0009607) include: the peptidoglycan recognition receptor (PGRP) SA involved in recognition of Gram+ bacterial peptidoglycan; CG9733, a protein with serine protease activity that is not known to play a role in Toll signaling or melanization [24]; CG13422 a protein with predicted glucosidase activity believed to play a role against Gram-bacteria; and the heat shock protein Hsp70 that is likely to be part of the stress response to pathogenic challenge [21,23,25-28]. More than half of all genes failing to induce in Relish mutants, belong to GO categories for humoral immune response (GO:0006959) and defense response (GO:0009607) (Table 1). Relish therefore appears to play a fairly specific role in regulating genes in this functional category. By contrast, less than a quarter of genes affected in *Dif* and *Dorsal* mutants fall in those two GO categories, and therefore these factors appear to play a relatively less specific role in mounting a humoral immune response (Table 1).

Dif mutants are unable to express significant levels of the AMPs *Defensin* and *Cecropin* as well as immune induced molecules IM1 and IM23 whose function in the immune response is not known [29,30]. CG13422 and CG9733 induced during an immune response, appear to require both Dif and Relish for their expression, along with 8 other genes (Figure 1). Heterodimerization between the Dif and Relish may be required for optimal expression of these genes. We find other immune induced genes that also require more than one Rel for expression. This suggests that other possible dimer combinations between Dorsal, Dif, and Relish may occur to produce functional transcription factors. *Dif* mutants also show a lowered expression of several genes that are not known to be involved in a defense response: CG3523, predicted

to be involved in fatty acid biosynthesis; Cytochrome P450 involved in steroid metabolism; and RSG7, important for regulating G-protein mediated signaling. Dif therefore maybe mediating more physiological changes associated with the immune response.

As previously reported, mutation of *dorsal* does not affect expression of known AMP genes. Dorsal was therefore believed not to play a role in the immune response. Genes affected by *Dif* or *Relish* mutation can induce from about 1.5 to 30 fold during infection. By contrast, none of the genes affected by a *Dorsal* mutation induced higher than 4.4 fold. This relatively smaller range of inducibility of Dorsal regulated immunity genes may also have resulted in an underestimation of Dorsal's importance in the immune response. However, in a broader genomic context the *dorsal* mutation does affect expression of a number of genes such as IM1, IM23, and CG6429 predicted to play a role in the defense response [21,26]. Like *Dif* however, *dorsal* also affects a number of proteins not directly associated with immunity: CG14762, predicted to be involved in cell adhesion, and Acp1, a structural constituent of the adult cuticle expression. *dorsal* mutants have decreased phenoloxidase activity and exhibit less melanization [31]. But our results with *dorsal* do not indicate significant changes in expression of genes associated with melanization, suggesting that these processes may not be primarily regulated at the transcriptional level. However, melanization is primarily regulated by a small number of hemocytes such as crystal cells. As a result, any change in the expression of melanization genes may not be detected above the overall expression patterns in the total RNA of the larvae.

While *dorsal* and *Dif* loss-of-function mutations affect the induction of fewer immunity genes compared to *relish*, their absence tends to up-regulate a relatively larger percentage of immunity genes by comparison. The Toll pathway regulates both Dorsal and Dif, [18] and loss of expression of either factor leads to the induction of some genes important for the Defense response including: Glutathione S-transferase D2; CG5550, which contains fibrinogen domains that may interact with extracellular matrix or receptors involved with the immune response; Peptidoglycan Recognition Receptor protein PGRP-SC2 that plays a role in the recognition of bacterial peptidoglycan and may suppress signaling through the imd pathway [32]; and Heat shock proteins HSP23 and HSP70 which are possibly induced as a response to stress during infection [21,23,33]. It is known that optimal expression of the Toll target gene, *Drosomycin* occurs 24 hrs post-infection, indicating that Toll pathway activation may affect an immune response days after infection. This is consistent with the observation that *dorsal* and *Dif* mutants show higher expression of some immune response genes suggesting that the Toll pathway may indeed be important for fine tuning later aspects of the immune response. On the other hand, in *relish* mutants, relatively few defense response genes are up-regulated, and instead we see no specific pattern by GO category. In sum, mutations in the three Rel proteins affect expression of distinct but overlapping groups of genes (Figure 1). This suggests that the use of multiple Rel proteins with distinct but overlapping functions may contribute to the complexity and regulation of distinct aspects of the immune response.

Ectopic Dorsal and Dif regulate different genes

Having examined loss of function mutations, we decided to ectopically express individual Rel proteins, to study their individual effect on global gene expression in the absence of infection. We used a heat shock (HS) driven promoter system to induce expression of transgenic Rel proteins in the absence of infection. For HS-*dorsal* and HS-*Dif*, exposure to heat shock resulted in a strong induction of the transgenic protein (Figure 2). For *Relish*, a heat shock driven Gal4 transcription factor was used to bind an upstream activation sequence (UAS) and drive *Relish* expression. This HS-Gal4;UAS-*Relish* system however did not produce reproducibly high levels of induction that would enable the study of *Relish* effects independent of *Dif* and *Dorsal* (Figure 2). Further, larvae that did express *Relish* transcript upon heat shock did not show a

corresponding induction of the established Relish target gene *Diptericin* (data not shown). There are reports that the truncated form of Relish, Rel^{ΔS29-S45} can induce Relish target genes *in vivo* and this suggests that signaling events leading to the cleavage of Relish may be important for its full activation [9]. The presence of additional cofactors, may also be necessary for optimal target gene transcription [9,34]. By contrast, the effect of Dorsal and Dif expression proved to be easier to study, because of robust transgenic expression and their simpler mechanism of regulation. These transcription factors are also interesting, because both of them are regulated by the same pathway and bound by the same IκB, Cactus. Yet there is clear evidence that they may be regulated differentially [31,35], and that once released they mediate transcription of different subsets of genes, as supported by our microarray studies. The distinct roles of Dif and Dorsal in the immune response were less apparent than that of Relish. Thus, the ectopic expression of these factors could provide insight into the possibility of redundancy between them, and help elucidate their respective roles in an immune response.

To examine the classes of genes activated by Dorsal and Dif *in vivo*, RNA was extracted from heat shock induced wildtype, HS-dorsal and HS-Dif larvae, labeled and hybridized onto cDNA microarrays constructed in our lab. These customized microarrays enable the study of 464 *Drosophila* genes selected from an extensive literature survey [22,23,26] and from our own microarray experiments, as being induced during the immune response. Genes that were significantly affected in HS-dorsal and HS-Dif larvae in comparison to wildtype were further classified based on available Gene Ontology information (Figure 3). Both Dorsal and Dif are capable of inducing a large number of genes involved in the humoral and defense responses. Over 25% of the genes induced when either Dorsal or Dif is expressed, belong to this category. Interestingly, ectopic Dorsal causes the induction of the AMP genes *Defensin*, *Diptericin*, *Attacin*, and *Metchnikowin*. *Dorsal* mutant larvae do not fail to induce any of these AMPs, suggesting that Dorsal may be acting redundantly with other Rel factors. Dorsal expression also induces components of the Toll pathway such as Dif, Cactus, and Pelle, suggesting a possible explanation for the induction of these components during infection (Supplementary Table 2). Ectopic Dif on the other hand notably induces Gram⁻ binding proteins (GNBP) 2 and GNBP3 which are involved with recognition of bacteria and fungi, and the AMPs *Attacin* and *Drosomycin* that have activity against these pathogens. Our results indicate that despite the fact that both Dorsal and Dif are regulated by the Toll pathway, they appear to play distinct roles as evidenced by their ability to upregulate distinct groups of immune genes.

Identifying Rel-specific target genes

In the past, target genes have been essential for identifying components of signaling pathways. Most components of the imd pathway for example, have been identified using genetic screens for mutations that failed to express its target gene *Diptericin* [36,37]. Similarly, the use of *Drosomycin* led to the seminal discovery of the importance of the Toll pathway in *Drosophila* immune responses [38]. *Drosomycin* expression has been the sole target gene used to assay the activation of the Toll pathway. However, *Drosomycin* has several limitations as a target gene. Other groups have reported that the imd pathway influences *Drosomycin* expression, and our data supports this finding. *relish* mutant larvae showed a significant lowering of *Drosomycin* expression compared to *dorsal* or *Dif* mutants alone. *In vitro* overexpression experiments have suggested that a Relish-Dif heterodimer is most effective at inducing *Drosomycin*, and this may explain these observations [12]. Further, *Drosomycin* is expressed at a high basal level and typically induces only up to two to six fold, often with peak induction at 24 hours after infection. With this long time frame, it is likely that pathways other than Toll may be activated and contribute to *Drosomycin* expression. Ideally, a Toll target gene would be highly inducible at an earlier time point for a more clear and specific readout of Toll pathway activation.

From our microarray data we looked for genes which might be regulated specifically by a particular Rel protein. A Dorsal target gene would, for example, not be expressed in *dorsal* mutant larvae, and induced when Dorsal is expressed ectopically. This regulation should also ideally be specific, such that a mutation in other Rel proteins should not affect its expression. From the microarray results we identified CG7214 as a putative Dorsal target gene, and CG15065 and CG13422 as putative Dif targets (Table 1). We used Q-PCR to confirm the expression of these genes in HS-dorsal and HS-Dif larvae (Figure 4). CG7214 failed to induce during ectopic Dorsal expression, while the putative Dif target genes CG15065 and CG13422 did induce to significant levels. Next, using Q-PCR we assayed the expression of these putative target genes in *Dif* and *dorsal* mutant flies (Figure 5). Flies with mutations in both the imd and Toll pathways (*imd;spätzle*), was used as a negative control while wildtype was used as a positive control. CG7214 was induced upon *M. luteus* infection, a known Gram⁺ bacterial activator of the Toll pathway. However, both *Dif* and *dorsal* mutant flies failed to express CG7214 suggesting that both proteins may be required for its induction, and that CG7214 is a non-specific target gene for Dorsal. On the other hand, both potential Dif targets were induced in wildtype *E. coli* and *M. luteus* infected flies within 2 hours but failed to be induced in *Dif* mutants. CG13422 is particularly attractive as a potential target, because it can be detected easily, inducing up to 20-fold within 2 hours after infection. Next, we analyzed up to 2kb upstream of all genes whose induction was affected in Rel mutants for putative NF- κ B sites using Target Explorer [39]. The presence of these sites suggests a possibility of direct binding and cis-activation of the gene by Dif, and we found sites matching predicted κ B motifs upstream of both CG15065 and CG13422 (Figure 6). These sites are also broadly conserved between related *Drosophila* species, suggesting that they may have been selected for through evolution. In combination with data from Figure 4 and 5, this suggests that CG15065 and CG13422 may be direct targets of Dif. We speculate that these target genes are directly bound by Dif, and specifically induced when Dif is activated in the nucleus. The use of these genes may therefore be used to assess Dif activation, and might help identify novel components of the Toll pathway in the future.

Conclusion

Here, we have presented the effect of Rel proteins on *Drosophila* immune gene expression *in vivo*, either when they are absent or when they are ectopically expressed. In this context, Relish plays a fairly focused role in mediating humoral and defense responses, while Dorsal and Dif are involved with inducing genes with a variety of functions. Some genes may be induced redundantly by the different Rels, and our ectopic expression experiments helped to identify genes that could be induced by Dorsal or Dif overexpression. Our data gives insight into possible heterodimer combinations that may be responsible for inducing different subgroups of genes. This data may help elucidate the distinct transcriptional roles of Rel family members. Because of significant conservation between Rel proteins, hypotheses generated based on their roles in *Drosophila* may be tested in other organisms. Finally, the identification and characterization of new target genes should facilitate the identification of novel components of the Toll pathway.

EXPERIMENTAL PROCEDURE

Larval staging and infection

Larvae were accurately staged to roughly 80 hours, as described [40]. The adult flies used were more than 5 days old. For infection, 1 ml of an overnight culture of *E. coli* DH5 α or *M. luteus* was spun down and resuspended in 1 ml of PBS. Approximately 0.1 μ l of this suspension was injected into flies using a pico-pump. For each sample, following injection with bacteria or PBS, 20 larvae or adults were homogenized and their RNA extracted using STAT-60 following the manufacturer's protocol.

Microarray experiments

Affymetrix microarray experiments were conducted using commercially available *Drosophila* GeneChips (Affymetrix, California). RNA was extracted from 50 larvae for each experimental replicate, repeated in triplicate for each genotype. Calculations were performed according to laboratory methods from the Affymetrix GeneChip manual. Genes which were induced greater than 2-fold with a p-value < 0.01 in at least 2 out of 3 replicates in wildtype *E. coli* injected larvae, were selected as induced during the immune response (GEO Acc. No. GSE5489).

The cDNA microarray comprised of 464 genes, selected based on previous results with Affymetrix chips, combined with genes selected from other published microarray studies as being induced during *Drosophila* immune responses [22,23,26]. We used Primer3 (http://www-genome.wi.mit.edu/genome_software/other/primer3.html) to design primers to amplify unique 200–600 bp regions of the selected genes (primer sequences available at GEO Acc. No. GPL4064). Fragments were amplified from whole genomic DNA of wildtype larvae in a 96 well format. Printing, hybridization and scanning of slides were performed with an Affymetrix 417 Arrayer and 418 Scanner (see <http://www.umbi.umd.edu/~cab/macore/macorestart.htm> for detailed protocols).

For the cDNA microarray experiments, RNA was extracted from a pooled sample of 20 larvae with STAT-60 buffer, according to the manufacturer's protocols (Isotex Diagnostics). The RNA was further purified using the Qiagen RNeasy purification kit, and directly labeled using Amersham Biosciences Cyscribe First-Strand Labeling Kit, according to manufacturer's protocols. The raw scanned image files were analyzed using Spotfinder (TIGR), and data normalization, quality assurance and control, filtering, and clustering was performed using MIDAS (TIGR) and MS-Excel [41]. Standard Deviation normalization and Lowess transformation was performed on the data using MIDAS (<http://www.tm4.org/midas.html>). The affected genes were then classified according to Gene Ontology, and the major groups are presented in Figure 3 (GEO Acc. No. GSE5469).

Quantitative PCR

The RNA was subjected to reverse transcription using Superscript II (Invitrogen) and the resulting cDNA was quantified by real-time PCR using LUX probes (Invitrogen) or SYBR Green (Applied Biosystems) on an ABI 5700 and 7300. Gene expression was normalized using RP49 as an endogenous control. The data presented in this paper has been further normalized to set uninjected or heat shock induced wildtype levels as the calibrator.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Figure 1. Venn diagrams of the numbers of genes significantly down-regulated (left) and up-regulated (right) in Rel mutant larvae

Among the 188 genes induced in wildtype, the number of genes that failed to induce to a significant level (left) in *dorsal*¹ (Green), *Dif*¹ (Orange), and *relish*^{E20} (Blue) larvae, or numbers of genes that were significantly up-regulated in these mutants (right) are shown.

Figure 2. Heat shock induces specific ectopic expression of Dorsal and Dif in HS-dorsal and HS-Dif larvae

Quantitative PCR measuring *dorsal*, *Dif*, and *relish* transcript levels in HS-dorsal, HS-Dif, and HS-Gal4;UAS-Relish larvae after exposure to 37°C for 1 hour relative to heat shock treated wildtype larvae. Error bars represent SD of at least three biological replicates, and (*) denotes statistically significant induction with p-value < 0.05 for two-tailed T-test.

Figure 3. Functional composition of genes significantly affected upon ectopic expression of Dorsal and Dif based on Gene Ontology

Genes significantly up-regulated (left), and down-regulated (right), in HS-Dif (top) and HS-dorsal (bottom) larvae after exposure to heat shock.

Figure 4. Q-PCR verification of expression of predicted target genes

Quantitative PCR showing CG7214, CG13422, and CG15065 transcript levels in HS-dorsal and HS-Dif larvae after exposure to heat shock. Error bars represent SD of at least three replicates and (*) denotes statistically significant induction with p-value < 0.05 for two-tailed T-test.

Figure 5. Expression of predicted target genes upon bacterial infection

Quantitative PCR measuring CG15065, CG13422, CG7214, Drosomycin, and Diptericin transcript levels in wildtype, *imd;spz*, *Dif*^d, and *dorsal*¹ / Deficiency J4. Flies are injected with *E. coli* or *M. luteus*, and harvested at 2, 6, or 24 hours after injection to examine gene expression. (*) denotes significant difference in expression from OR flies with same infection and time point with a p-value < 0.05 for two-tailed unpaired T-test.

CG15065

yakuba
mojavensis
melanogaster
pseudoobscura

```
GAAACA TTTTTC CTGGGGCATTTCCTTGTGGAACAACCTTGT-----
GAAACA TTTTTC TAGGGCATTTCCTGTG GACTTGGAACGACCTTGTACTTGAA
CATTTC TTTTGTAGGGCATTTCCTTGTG GACTGGGCACAACCTTGTG-----
CATTTC GTTTTGTAGGGATTTCCTTGTG GACTTGGCACGACTATGTG-----
```

Putative NF-κB site

CG13422

melanogaster
pseudoobscura
yakuba
mojavensis

```
GAGGTATGTGGTTGGGCAGGGGGATTCCCAGAGAATCGATGTCAACGGCTCCAACGG
GAGGCATGTGGTCGGTCAGGGGGATTCCCAGAGAATAGATGTCAACGGCTCCAACGG
GAGGCATGTGGTCGGGCAGGGGGATTCCCAGAGAATCGATGTCAATGGCTCCAACGG
GAGGCATGTGGTTGGGCAGGGGGATTCCCAGAAATCGACGTGAACGGCTCAACGG
```

Putative NF-κB site

Figure 6. Conservation of putative NF-κB binding site upstream of CG15065 and CG13422 between related *Drosophila* species

The sites were identified using Target Explorer, 198 bp (CG15065) and 119 bp (CG13422) upstream of their respective start sites. Clustal alignments of available sequences from related species show conservation of the binding site.

Table 1
Selected list of genes regulated by Dorsal, Dif, and Relish

188 Genes were significantly induced in wildtype larvae upon *E. coli* infection. Among them, genes which were significantly affected in specific Rel mutants are listed. Genes affected by a specific Rel mutation are grouped together, and pie charts (Column 1) represent the relative functional compositions of these genes based on available Gene Ontology information. (Columns 2-4) Gene, wildtype induction, and wildtype average intensity are provided. (Columns 5-7) Ratios of mutant final expression intensity divided by wildtype intensity are presented as ratios for Dorsal, Dif, and Relish mutant lines. If the ratio is more than one standard deviation below the mean ratio for all genes in the category, it is colored Green to indicate significant reduction. Red denotes significant expression, with ratios over 1 SD above the wildtype. (Columns 8-9) The fold change of genes when Dorsal or Dif are ectopically expressed. The values are colored Green if they represent a significant reduction and Red if there is significant induction.

Gene	Wildtype Induction	Wildtype Intensity	Ratio induced in Dorsal mutant	Ratio induced in Dif mutant	Ratio induced in Relish mutant	Fold change in HS-Dorsal	Fold Change in HS-Dif
Genes requiring Dorsal expression							
CG18239	2.04	11145.40	0.04	0.09	0.03	1.08	1.22
IMI	3.23	6993.00	0.07	0.04	0.31	0.89	1.02
CG14419	2.12	4529.70	0.13	0.29	0.22	1.04	0.63
CG14481	2.43	2901.70	0.13	0.62	0.43	0.40	0.29
CG14499	1.74	908.20	0.15	0.45	0.15	0.92	0.98
Acp1	1.58	15977.20	0.17	0.44	0.42	0.83	0.92
CG17104	1.77	793.20	0.18	0.64	0.77	1.11	1.23
IM23	3.64	942.10	0.19	0.15	0.56	1.15	1.13
Ccp84Ab	1.72	1184.80	0.20	1.35	0.72	1.02	1.02
CG6429	4.38	1779.20	0.21	0.77	0.37	0.74	1.42
Faa	1.57	1413.10	0.22	1.08	0.62	0.80	1.16
CG7214	1.45	9524.80	0.24	0.64	0.59	1.39	0.74
CG14762	1.75	1105.10	0.26	0.43	0.12	1.02	1.00
CG14850	1.98	27734.70	0.32	0.12	0.76	1.09	1.16
Genes requiring Dif Expression							
CG17105	1.44	11542.10	2.51	0.02	2.81	1.10	1.00
CG13135	2.99	8609.40	1.47	0.02	0.34	1.03	1.05
IMI	3.23	6993.00	0.07	0.04	0.31	0.89	1.02
CG18067	3.45	8295.50	1.09	0.08	0.94	0.72	1.25
CG18239	2.04	11145.40	0.04	0.09	0.03	1.08	1.22
CG14850	1.98	27734.70	0.32	0.12	0.76	1.09	1.16
BcDNA:GH07626	1.75	3850.20	0.73	0.12	1.45	0.85	0.93
CG13461	1.64	3817.30	2.10	0.13	0.59	1.36	1.03
Def	7.07	1644.90	1.02	0.14	0.04	1.08	0.95
CG13422	4.26	3048.90	2.20	0.14	0.23	1.03	1.43
CG15065	3.12	14514.70	1.74	0.14	0.64	0.94	1.35
IM23	3.64	942.10	0.19	0.15	0.56	1.15	1.13
CG15067	2.23	1420.70	1.20	0.16	0.20	0.51	0.98
Cypl1	1.93	1026.40	1.60	0.17	1.10	0.96	0.78
CG9733	17.81	971.30	0.97	0.18	0.19	1.11	1.18
CG6906	1.77	3046.50	0.83	0.21	0.62	1.31	0.88

21 Genes Total

- Unknown
- Others
- Proteolysis and Peptidolysis
- Cell Proliferation
- DNA repair
- Defense response
- Humoral response
- Metabolism

26 Genes Total

Gene	Wildtype Induction	Wildtype Intensity	Ratio induced in Dorsal mutant	Ratio induced in Dif mutant	Ratio induced in Relish mutant	Fold change in HS-Dorsal	Fold Change in HS-Dif
CG8087	1.38	9746.40	1.25	0.24	0.40	0.88	0.88
CG14419	2.12	4529.70	0.13	0.29	0.22	1.04	0.63
CecB	31.91	4981.10	1.33	0.29	0.08	0.70	0.81
Genes requiring Relish Expression							
Diptericin	33.85	12251.30	1.70	1.39	0.01	1.00	0.91
CecA2	55.02	4284.20	2.37	0.61	0.02	1.11	1.16
CG18239	2.04	11145.40	0.04	0.09	0.03	1.08	1.22
Def	7.07	1644.90	1.02	0.14	0.04	1.08	0.95
CecC	11.62	3822.00	1.55	0.60	0.04	0.90	0.69
DptB	7.07	12703.20	1.90	1.58	0.06	1.44	0.98
Rel	5.78	4654.10	1.15	1.02	0.06	1.09	0.96
AtfD	2.62	2517.40	1.11	0.99	0.07	0.61	0.40
CecB	31.91	4981.10	1.33	0.29	0.08	0.70	0.81
CecA1	15.93	9810.40	2.80	0.61	0.08	1.02	0.99
CG14762	1.75	1105.10	0.26	0.43	0.12	1.02	1.00
CG14499	1.74	908.20	0.15	0.45	0.15	0.92	0.98
Drosomycin	8.97	8581.90	2.06	0.49	0.15	1.11	1.73
Hsp70 BC	5.92	854.20	1.19	7.90	0.18	1.41	1.04
CG9733	17.81	971.30	0.97	0.18	0.19	1.11	1.18
CG15067	2.23	1420.70	1.20	0.16	0.20	0.51	0.98
CG9080	3.05	11125.20	3.36	2.03	0.22	1.86	1.21
CG14419	2.12	4529.70	0.13	0.29	0.22	1.04	0.63
CG13422	4.26	3048.90	2.20	0.14	0.23	1.03	1.43
PGRP-SA	2.69	9727.20	0.63	0.67	0.27	3.25	1.00
Genes upregulated in Dorsal mutants							
Cyp4e3	1.49	1855.50	10.14	0.62	1.81	1.09	0.82
PGRP-SC2	1.37	6088.40	4.31	3.01	5.21	0.99	1.08
Hsp23	1.94	2226.00	3.77	6.00	1.12	1.03	1.43
CG9080	3.05	11125.20	3.36	2.03	0.22	1.86	1.21
CG4740	18.72	7473.40	3.32	2.10	0.35	1.87	1.56
Milk	7.99	8593.60	3.07	1.50	0.41	1.63	0.89
CecA1	15.93	9810.40	2.80	0.61	0.08	1.02	0.99
Genes upregulated in Dif mutants							
Hsp70 BC	5.92	854.20	1.19	7.90	0.18	1.41	1.04
Hsp23	1.94	2226.00	3.77	6.00	1.12	1.03	1.43
CG12505	1.63	8898.30	1.24	3.36	0.44	0.90	1.13
GstD2	1.78	9626.40	0.59	3.31	1.64	1.81	1.16
CG5550	2.96	3754.10	1.92	3.05	0.85	1.00	0.00
PGRP-SC2	1.37	6088.40	4.31	3.01	5.21	0.99	1.08
Genes upregulated in Relish mutants

28 Genes Total

19 Genes Total

16 Genes Total

22 Genes total	Gene	Wildtype Induction	Wildtype Intensity	Ratio induced in Dorsal mutant	Ratio induced in Dif mutant	Ratio induced in Relish mutant	Fold change in HS-Dorsal	Fold Change in HS-Dif
	PGRP-SC2	1.37	6088.40	4.31	3.01	5.21	0.99	1.08
	CG17105	1.44	11542.10	2.51	0.02	2.81	1.10	1.00
	InR	3.16	310.20	1.87	2.29	2.58	1.02	0.90
	CG13686	2.28	59.10	2.29	1.47	2.30	1.15	1.40
	CG13905	3.10	3017.20	2.12	1.91	2.28	1.20	1.08