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A Member of the p38 Mitogen-Activated Protein Kinase Family Is Responsible for Transcriptional Induction of *Dopa decarboxylase* in the Epidermis of *Drosophila melanogaster* during the Innate Immune Response▽†

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***Drosophila* innate immunity is controlled primarily by the activation of IMD (immune deficiency) or Toll signaling leading to the production of antimicrobial peptides (AMPs). IMD signaling also activates the JUN N-terminal kinase (JNK) cascade, which is responsible for immune induction of non-antimicrobial peptide immune gene transcription though the transcription factor AP-1. Transcription of the *Dopa decarboxylase* (*Ddc*) gene is induced in response to gram-negative and gram-positive septic injury, but not aseptic wounding. Transcription is induced throughout the epidermis and not specifically at the site of infection. *Ddc* transcripts are detectible within 2 h and remain high for several hours following infection with either gram-negative or gram-positive bacteria. Using *Ddc*-green fluorescent protein (GFP) reporter gene constructs, we show that a conserved consensus AP-1 binding site upstream of the *Ddc* transcription start site is required for induction. However, neither the Toll, IMD, nor JNK pathway is involved. Rather, *Ddc* transcription depends on a previously uncharacterized member of the p38 mitogen-activated protein kinase family, p38c. We propose that the involvement of DDC in a new pathway involved in *Drosophila* immunity increases the levels of dopamine, which is metabolized to produce reactive quinones that exert an antimicrobial effect on invading bacteria.**

Most animal species exhibit the ability to ward off infection. While mammals employ a combination of adaptive and innate immune responses, the majority of organisms use only innate immunity to combat infection. The genetic and molecular techniques available for *Drosophila melanogaster* have made it an exceptional model for innate immune research (31). *Drosophila* immunity combines humoral and cellular responses to effect a strong resistance to many microorganisms. The three mechanisms contributing to this resistance are phagocytosis of invading organisms by hemocytes; blood clotting, melanin formation, and opsonization; and transient synthesis of antimicrobial peptides (AMPs) at both the wound site and in the fat body.

Drosophila lives in decaying and fermenting matter and therefore is exposed to a multitude of bacteria, fungi, and viruses that it must defend itself against. The ability to combat natural and septic infections by gram-negative and gram-positive bacteria has been the focus of much study in the last several years. The work has centered on the humoral defense response, involving the synthesis and release of AMPs from the fat body into the hemolymph. Transcription of different but overlapping sets of approximately 20 AMPs (32, 43) is elicited by gram-negative bacteria upon activation of the IMD (immune deficiency) pathway (12, 44, 46) and gram-positive bacteria and fungi following induction of the Toll pathway (45, 68). Each pathway acts through its respective NF-κB transcription factor(s); the Toll pathway uses Dorsal and DIF (33, 50, 55, 61, 66), while the IMD pathway activates Relish (19, 28).

Immune activation of the IMD pathway also causes the induction of the mitogen-activated protein kinase (MAPK) cascade, known as the JUN N-terminal kinase (JNK) pathway, due to a bifurcation at the transforming growth factor (TGF)-activated kinase, TAK1 (9, 73, 80). In *Drosophila*, the MAPK kinase (MAPKK), Hemipterous (HEP) (23), activates the JNK, Basket (BSK) (9), which can then phosphorylate the heterodimeric transcription factor AP-1 (40, 64), which is composed of a FOS subunit and a JUN subunit (59, 87). Phosphorylation activates AP-1 and causes induction of JNK response genes. JNK signaling is required for proper wound healing in the adult epidermis (21, 63) and the wing disc (8). JNK signaling also activates the transcription of many proteins involved in cytoskeleton remodeling (9), consistent with its role in hemocyte activation in the cellular immune response (86). A role for JNK signaling in AMP gene expression has also been proposed (18, 35); however, further research is needed since JNK repression of AMP synthesis has also been proposed (38).

In addition to the JNK pathway, two other conserved MAPK pathways have potential roles in *Drosophila* immunity and wound healing. The first, the extracellular signal-regulated kinase (ERK) pathway, has been implicated in the *Drosophila* embryonic wound response (49). The second is the p38 MAPK family, which has two characterized members (p38a and p38b) that have been implicated in attenuation of the immune response (27). p38 MAPKs are activated by dual phosphorylation

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by a MAPKK at a characteristic TGY site (51). This site differs from the dual-phosphorylation TPY and TEY motifs of JNK and ERK, respectively.

The primary layer of defense against infection involves the barrier epithelia of the epidermis, including the hypoderm that lies beneath the cuticle, and the epithelial tissues of the trachea and gut. The barrier epithelia are responsible for localized production of AMPs at the site of septic injury (20, 79). While formation of a melanin clot at the wound site is not specifically an immune response, it is essential to prevent hemolymph loss following septic injury. An additional benefit is that the rapid production of the melanized clot entraps bacteria and promotes killing. The melanin produced at the clot site is a direct consequence of the catalytic activation of the zymogen prophenol-oxidase (5), that is stored in the crystal cells (53, 65), to produce active phenol oxidase (PO). PO uses tyrosine to produce the quinones that react nonenzymatically to form melanin (53, 54). Clots form without the action of PO (5, 71); however, PO is required for clot hardening (5, 36). An additional potential role for melanin production at the wound site involves the formation of reactive oxygen species and by-products of melanin production that have cytotoxic antimicrobial properties (58). Interestingly, the JNK cascade is necessary for crystal cell rupture following injury, which leads to melanization at the wound site (4).

In addition to the role of PO in wound healing, two other genes, *pale* (*ple*) and *Dopa decarboxylase* (*Ddc*) (83), have been shown to be essential to wound healing in the *Drosophila* embryo (49). These genes encode the enzymes tyrosine hydroxylase (TH), which converts tyrosine to dihydroxyphenylalanine (Dopa), and Dopa decarboxylase (DDC), which converts Dopa to dopamine (DA). The neural role of DA is well established; however, DA is also metabolized to produce melanin and sclerotin in epidermal tissues (2, 83), which leads to tanning of the pupal case and adult cuticle and pigmentation of the pharate adult epidermis (81). The transcription factor Grainy-head is responsible for *Ddc* induction in the embryonic wound response, and it acts through a binding site just upstream of the transcription start site (49). However, deletion of three AP-1 sites and a CREB (an ERK-inducible transcription factor) binding site upstream of *Ddc* also eliminates expression of *Ddc* during the wound response, indicating a potential role for the JNK or ERK pathways in this induction. Consistent with the role of a MAPK in *Ddc* transcriptional induction, injection of the MAPK inhibitor PD98059 into embryos reduced the induction of a *Ddc*-GFP reporter after aseptic injury.

Evidence suggests that *Ddc* plays a role in the *Drosophila* innate immune response in larvae and adults. Defense against the parasitic wasp *Leptopilina boulardii* requires the production of a melanized capsule that envelops the egg and eventually kills it. Melanotic encapsulation requires the actions of specialized blood cells called lamellocytes (53, 54). Nappi et al. (57) showed that the melanization response was severely compromised in Ddc^{ts2} flies, demonstrating the need for functional DDC in the encapsulation response. *Ddc* transcription was induced in larvae of *Tenebrio molitor* infected with *Escherichia coli* (39), and this corresponded to increased DDC protein and activity levels. In addition, *N*-β-alanine (NBAD) synthase, an enzyme that converts DA to the sclerotin precursor, NBAD, is induced in the epidermis of *T. molitor* and *Ceratitis capitata* following *E. coli* infection (70). NBAD has potent antimicrobial effects, which can be eliminated by the addition of antioxidants, suggesting that NBAD elicits its killing effects because of spontaneous conversion to cytotoxic quinones. Together, these results suggest a role for DA metabolism in the insect innate immune response. Consistent with this prediction, microarrays performed on flies and SL2 cell culture have identified *Ddc* as one of the genes induced after both gram-positive and gram-negative bacterial infection, but not fungal infection (9, 17, 34).

In this paper, we investigate the role of *Ddc* in the *Drosophila* innate immune response to bacterial infection. We find that *Ddc* is induced upon septic injury, but not aseptic wounding of larvae and adults, and this response is dependent on a conserved AP-1 consensus binding site upstream of the *Ddc* transcription start site. However, JNK signaling is not responsible for induction of *Ddc* transcription; rather, an uncharacterized p38 MAPK, p38c, is responsible. Induction of *Ddc* occurs throughout the epidermis and is not restricted to the wound site. We propose that DDC activity is required in the epidermis for the synthesis of DA that is metabolized to produce reactive quinones that exert killing effects on invading bacteria.

MATERIALS AND METHODS

Fly stocks, crosses, and infection of organisms. The stocks used in this study, their sources, and their purposes are shown in Table S1 in the supplemental material (47). All stocks were maintained on a standard cornmeal/molasses medium at room temperature. To obtain Ddc^{ts2} *cn*/*rdo hk* Ddc^{n7} *pr cn* and Ddc^{ts2}*cn*/*Df(2L)130 pr cn* flies, Ddc^{ts2} *cn*/*CyO* female virgins were crossed to *rdo hk* Ddc^{n7} *pr cn*/*CyO* or *Df(2L)130 pr cn*/*CyO* males, respectively. The progeny were raised at the 18°C permissive temperature. Mutants were identified by their wild-type wings, separated from their curly-winged wild-type siblings, and transferred to the 25°C restrictive temperature for 4 days prior to infection.

The following P-element excision scheme was undertaken to obtain mutants and revertants of $p38c^{KG05834}$, a strain carrying a P-element marked with a mini-w^+ gene: (i) *w*; $p38c^{KG05834}$/*TM3*, *Sb* ♀s × *w*/*Y*; *Sb e* Δ*2-3*/*TM6*, *Ubx e* ♂s → (ii) *yw*; *PSal89D, Sb*/*TM6, Ubx e* ♀s × *w*/*Y*; $p38c^{KG05834}$/*Sb e* Δ*2-3* ♂s (red eyed) → (iii) *yw*; *PSal89D*, *Sb*/*TM6*, *Ubx e* ♀s × *yw*/*Y*; *p38c*/TM6*, *Ubx e* ♂s (* indicates a P-element excision, identified by the presence of white eyes) → (iv) *yw*; *p38c*/TM6*, *Ubx e* ♀s × *yw*/*Y*; *p38c*/TM6*, *Ubx e* ♂s → (v) homozygous or balanced stock.

The region of *p38c* spanning the P-element excision, from the homozygous P-element excision flies, was PCR amplified, cloned into pGEM-T Easy (Promega), and sequenced.

All larvae that were infected were in the mid-third instar, during which time no epidermal *Ddc* transcription is detectible (M. M. Davis, unpublished observation). Adults that were infected were between 4 and 6 days old, a time when epidermal *Ddc* transcripts are at minimal levels (14). At least 10 adults and 30 larvae of each genotype were septically injured with a sharpened tungsten needle that was dipped into the pellet obtained from a saturated culture of *E. coli* DH5α or *Staphylococcus aureus* ATCC 6538-P. For heat shock experiments, organisms were heat shocked for 1 h at 37°C and allowed to recover at room temperature for 1 h before being septically injured. Adults were injured by being poked in the thorax immediately below the wing, and larvae were wounded in the posterior end. Adults were allowed to recover in a vial containing standard *Drosophila* medium, and larvae recovered on a piece of sterile water-soaked Whatman paper in a petri dish to prevent desiccation after injury. With the exception of the time course shown in Fig. 5, organisms were allowed to recover for 4 to 6 h following infection. Only the organisms that were alive at harvesting were frozen for subsequent use.

Plasmid construction and the creation of transgenic lines. The P-element plasmids P[*Ddc*-GFP]PH, P[*Ddc*-GFP]SH, P[*Ddc*-GFP]BH, and P[*Ddc*-GFP]EH (Fig. 1) have been described elsewhere (15). The P[*Ddc*]PHΔBE, P[*Ddc*]PHΔBE1, P[*Ddc*]PHΔBE2, P[*Ddc*]PHΔBE3, P[*Ddc*]PHmutNFκB, and P[*Ddc*]PHmutAP-1 constructs in pBluescript SK(+) were all created by inverse PCR using phosphorylated primer pairs (see Table S2 in the supplemental material, which lists the PCR primers and oligonucleotides used in this study) ΔBE3-F and ΔBE1-R, ΔBE1-F and

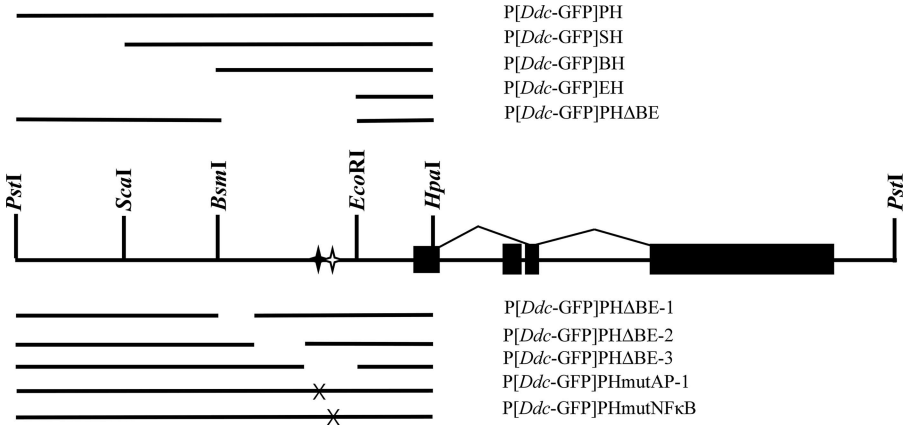

FIG. 1. Schematic diagram of the P[*Ddc*-GFP] reporter constructs used in this study. The epidermis-specific splice pattern shown above the line is situated on the 7.6-kb PstI restriction fragment that is sufficient for proper developmental expression of *Ddc*. The second exon is nerve specific. The sequence that drives GFP expression is depicted in the lines above and below the diagram of the *Ddc* genomic region. Relevant restriction enzyme sites, used to create the larger deletions, are shown. The black and white stars represent the conserved AP-1 and NF-κB sites, respectively; X indicates an induced mutation. The P[*Ddc*-GFP]PHmutNFκB construct contains a 5-bp mutation in the putative NF-κB binding site, and the P[*Ddc*-GFP]PHmutAP-1 construct contains a 5-bp mutation in the putative AP-1 site.

ΔBE1-R, ΔBE2-F and ΔBE2-R, ΔBE3-F and ΔBE3-R, NFκB-F and NFκB-R, and AP1-F and AP1-R, respectively, on PH in pBluescriptSK(+). The resulting products were gel purified and ligated intramolecularly to create the respective plasmids. The breakpoints of the deletions and site specific mutations were all confirmed by sequencing. The *Ddc* region contained within each plasmid was then liberated by digestion with BamHI and KpnI and cloned into similarly digested pGreen Pelican (3) to create P[*Ddc*-GFP]PHΔBE, P[*Ddc*-GFP]BE-1, P[*Ddc*-GFP]BE-2, P[*Ddc*-GFP]BE-3, P[*Ddc*-GFP]mutNFκB, and P[*Ddc*-GFP]mutAP-1 (Fig. 1).

To create the P[*Ddc*-RNAi]pSymp and P[*Ddc*-RNAi]pWIZ constructs, a 490-bp region of the *Ddc* coding sequence within the 4th exon was amplified with the DDC-NotIF and DDC-NotIR and DDC-XbaIF and DDC-XbaIR primer pairs. The DDC-NotI primers add NotI sites to facilitate cloning into pSymp (22), and the DDC-XbaI primers add XbaI sites to facilitate cloning into pWIZ (42). Both pSymp and pWIZ express the cloned region under the control of a UAS promoter, facilitating Gal4 induction of the transgene. The PCR fragments were gel purified and cloned into pGEM-T Easy (Promega) to create *Ddc*-NotI in pGEM-T Easy and *Ddc*-XbaI in pGEM-T Easy. The *Ddc* fragment was then liberated from *Ddc*-NotI in pGEM-T Easy by NotI digestion and cloned into similarly digested pSymp to create P[*Ddc*-RNAi]pSymp. To create P[*Ddc*-RNAi]pWIZ, the XbaI fragment from *Ddc*-XbaI in pGEM-T Easy was first cloned into the AvrII site of pWIZ and then cloned again into the NdeI site of the resulting plasmid. Both plasmids were sequenced to confirm the orientation of the *Ddc* fragment inserted into each site.

DNA solutions (0.4 μg/μl DNA in 0.5× phosphate-buffered saline and 10% glycerol) containing transgenic constructs were injected into embryos of the genotype *w*; *Sb e* Δ*2–3/TM6, Ubx e*. Surviving adults were crossed to *yw* flies, and progeny with the w^+ phenotype were collected and mated to produce homozygous or balanced stocks.

RNA extraction and RT-PCR. RNA was extracted from pools of organisms using TRIzol, treated with amplification-grade DNase I (Invitrogen) according to manufacturer's instructions, and then extracted with phenol-chloroform and then chloroform. Semiquantitative *Ddc* and *Ddc*-GFP reverse transcription PCRs (RT-PCRs) were performed as detailed elsewhere (14, 15). Synthesis of *p38c*-specific cDNA was initiated using p38c-1 (see Table S2 in the supplemental material) on 150 ng of isolated RNA. The first-strand reaction was co-reverse transcribed with RPL-32-1, a primer specific for the ribosomal gene *RpL32*, which served as a loading control. A 3-μl aliquot of the resulting cDNA mixture was combined with *p38c* forward and reverse primers (p38c-2F and p38c-2R) and amplified for six cycles. The program was then stopped, *RpL32* gene-specific primers (RPL32-F and RPL32-R) were added, and the program was allowed to continue for 23 more cycles. After an initial denaturing step of 2 min, the final PCR conditions for *p38c* consisted of 29 cycles of 95°C for 1 min, 60°C for 1 min, and 73°C for 1 min.

Under these conditions, the amount of each product was proportional to input RNA concentration (see Fig. S1 in the supplemental material). The band intensity was quantified using Image J (1). Using 150 ng of input RNA, the amount of *Ddc* and *Ddc*-GFP product was proportional to the number of cycles for 24 to 27 cycles, the amount of *RpL32* product was proportional to the number of cycles for 22 to 25 cycles, and the amount of *p38c* product was proportional to the number of cycles for 27 to 30 cycles.

For the nonquantitative RT-PCR on the *p38a* and *p38c* transcripts (see Fig. 9B and C), greater than 1 μg of total RNA was reversed transcribed using Superscript II reverse transcriptase (Invitrogen) using the primers p38a-R and p38c-1, respectively. The cDNA was then used as a template for a PCR using the p38a-F and p38a-R primer pair for *p38a* and the p38c-2F and p38c-2R primer pair for *p38c*. The final PCR was 30 cycles of 95°C for 1 min, 60°C for 1 min, and 73°C for 1 min.

Quantitative real-time PCR. RNA (2 μg) was reverse transcribed with random primers (Applied Biosystems). The cDNA mixture was diluted 1:20, and a 2.5-μl aliquot was used as a template for quantitative real-time PCR using *Ddc* (DDC-RT1 and DDC-RT2)- or *RpL32* (RPL32-RT1 and RPL32-RT2)-specific primer pairs and ABI Power Sybr green PCR master mix according to the manufacturer's instructions (Applied Biosystems). Applied Biosystems StepOne software was used for quantifying the transcripts.

Production of recombinant proteins. The constructs made up of dFRA (*Drosophila fos*) and dJRA (*Drosophila jun*) in pBluescript KS(+) were a generous gift from Robert Tjian. These plasmids were used as templates for in vitro transcription and translation using the TNT T3 coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions.

Electrophoretic mobility shift assays. Mobility shift assays were carried out using a 26-bp double-stranded oligonucleotide probe with a 2-bp extension. The 28-base single-stranded oligonucleotides were individually end labeled with [γ-^{32}P]ATP using T4 kinase (Invitrogen) according to the manufacturer's instructions and annealed. The wild-type probe was obtained by annealing the oligonucleotide pair AP1-A and AP1-B (see Table S2 in the supplemental material), and the probe containing the mutated AP-1 site was obtained by annealing AP1M-A and AP1M-B.

The recombinant proteins or reticulocyte lysate mix was incubated in binding buffer (20 mM HEPES-KOH [pH 7.9], 50 mM KCl, 4 mM $MgCl_2$, 4 mM spermidine, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.05% NP-40 and 20% glycerol) in the presence of 2 μg of the nonspecific competitor poly(dI-dC) (Amersham) and specific cold competitor, where applicable, for 5 min at room temperature. Two nanograms of radiolabeled probe was added to the reaction mixture and allowed to bind at room temperature for 15 min. Binding mixtures were fractionated on a 6% polyacrylamide gel in 0.5× Tris-borate-EDTA running buffer, and the products were visualized by autoradiography.

RESULTS

***Ddc* transcription is induced in the *Drosophila* innate immune response.** Microarray studies of *Drosophila* immunity

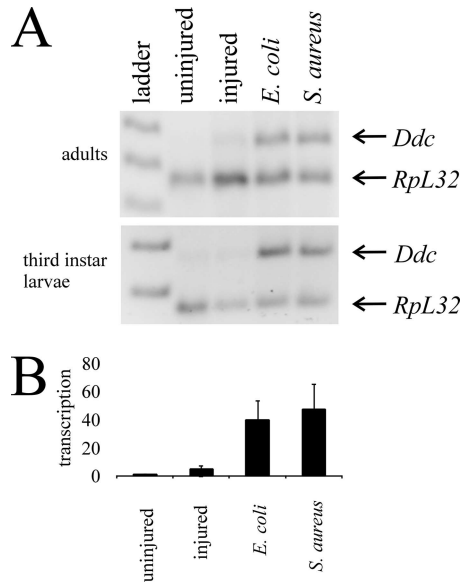

FIG. 2. Immune induction of *Ddc* transcription following septic injury. (A) Semiquantitative RT-PCR was carried out on RNA obtained from adult flies and mid-third-instar larvae. Gene-specific transcripts in this and subsequent figures were separated on a 2% agarose gel and are shown with a co-reverse-transcribed and PCR-amplified *RpL32* loading control. Transcripts from organisms septically injured with *E. coli* (lane 4) or *S. aureus* (lane 5) are compared to aseptically injured (lane 3) or uninjured (lane 2) organisms. In this and subsequent figures, the 1-kb Plus DNA ladder (Invitrogen) was used as a size standard. (B) Quantitative real-time PCR of *Ddc* transcripts in the same samples confirms that *Ddc* is induced following septic injury with *E. coli* and *S. aureus*, but not following aseptic injury.

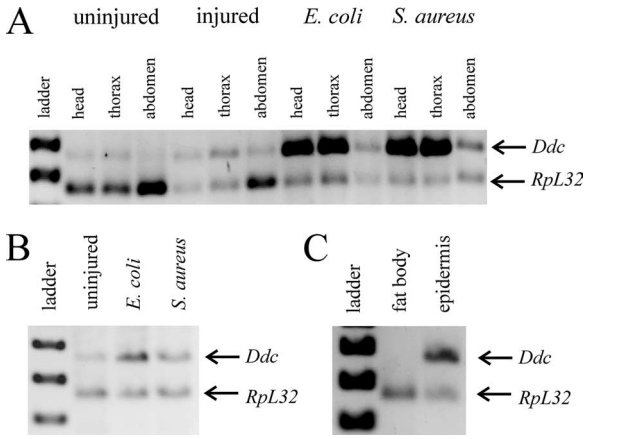

FIG. 3. *Ddc* transcription is induced in the epidermis. (A) Semiquantitative RT-PCR from RNA isolated from heads, thoraxes, and abdomens of uninjured, aseptically injured, and septically injured adult flies. (B) RT-PCR of RNA isolated from mid-third-instar dom^{K08108} larvae infected with *E. coli* or *S. aureus*. (C) RT-PCR on RNA isolated from fat body and epidermal tissues dissected from mid-third-instar larvae infected with *E. coli* or *S. aureus*.

have identified *Ddc* as an immune-inducible gene (9, 17, 34). To confirm that *Ddc* transcription is induced by bacterial infection, we performed semiquantitative RT-PCR on uninjured, aseptically injured, and septically injured mid-third-instar larvae and adult flies, harvested 4 h after treatment (Fig. 2A). The *Ddc* forward and reverse PCR primers are anchored in the first and third exons of *Ddc*, respectively, and the PCR conditions are such that we never amplify the larger product that contains the nerve-specific second exon (Fig. 1). Transcription of *Ddc* is induced when larvae or adults are infected with *E. coli* (gram negative) or *S. aureus* (gram positive) and when organisms are aseptically injured with a large needle (data not shown); however, little *Ddc* transcript is detectible when organisms are aseptically injured with a small needle or left untreated (Fig. 2A). We find that if organisms are septically injured with a small needle, *Ddc* transcription is induced, whereas if that needle is used for aseptic injury, *Ddc* transcription is absent. We attribute *Ddc* induction following aseptic injury with a large needle to infection with natural flora on the *Drosophila* cuticle.

To confirm that our method of semiquantitative RT-PCR was valid, we used real-time PCR to amplify the same samples examined in Fig. 2A. Similarly, we found that *Ddc* transcription was induced following septic injury with either *E. coli* or *S. aureus*, but *Ddc* transcript was not detected following aseptic injury or in control organisms (Fig. 2B). This confirms the validity of our semiquantitative RT-PCR analyses, which are presented in the remainder of this article.

Systemic *Ddc* transcription is localized to the epidermis. Having confirmed that *Ddc* is induced in the immune response, we next set out to identify which tissues express *Ddc*. To do this, we aseptically and septically injured adult flies and 4 h later separated their heads, thoraxes, and abdomens for RNA isolations. We find that *Ddc* transcription is induced strongly in the head and thorax and less so in the abdomen of the flies that were septically injured with either *E. coli* or *S. aureus* (Fig. 3A). This induction was absent in flies that were uninjured or aseptically injured. This suggests that *Ddc* transcription is induced throughout the organism and is not restricted to the site of infection.

Since *Ddc* transcription is induced throughout the adult organism, the three most likely locations of transcriptional induction are in the hemocytes, the fat body, or the epidermis. In order to determine if *Ddc* transcription is induced in the hemolymph, we examined the induction of *Ddc* transcription in larvae of *domino* mutants that fail to develop functional hemocytes (10). We were unable to assess *Ddc* induction in adult dom^{K08108} flies, as these mutants die at pupariation when large numbers of hemocytes are necessary for phagocytosis at the onset of metamorphosis. However, we find that *Ddc* transcription is induced normally in dom^{K08108} larvae (Fig. 3B, lanes 3 and 4) and that these mutants appear to have a high basal level of *Ddc* transcription (lane 2), likely due to chronic infection caused by the lack of hemocyte-derived cellular defenses.

To eliminate the possibility that the fat body cells could adhere to the epidermis preparations, we obtained epidermal tissue from larvae. At this stage, the fat bodies are much more defined than in adults (41) and were easily separated from the epidermis. Tissues were isolated by dissection from mid-third-instar larvae 4 h after septic injury, and semiquantitative RT-PCR was performed on the RNA obtained from these samples. The result clearly shows that *Ddc* transcription is induced in the epidermal tissues and not the fat body (Fig. 3C).

Reduction of DDC activity has no effect on adult survival following infection. To determine if DDC activity within the

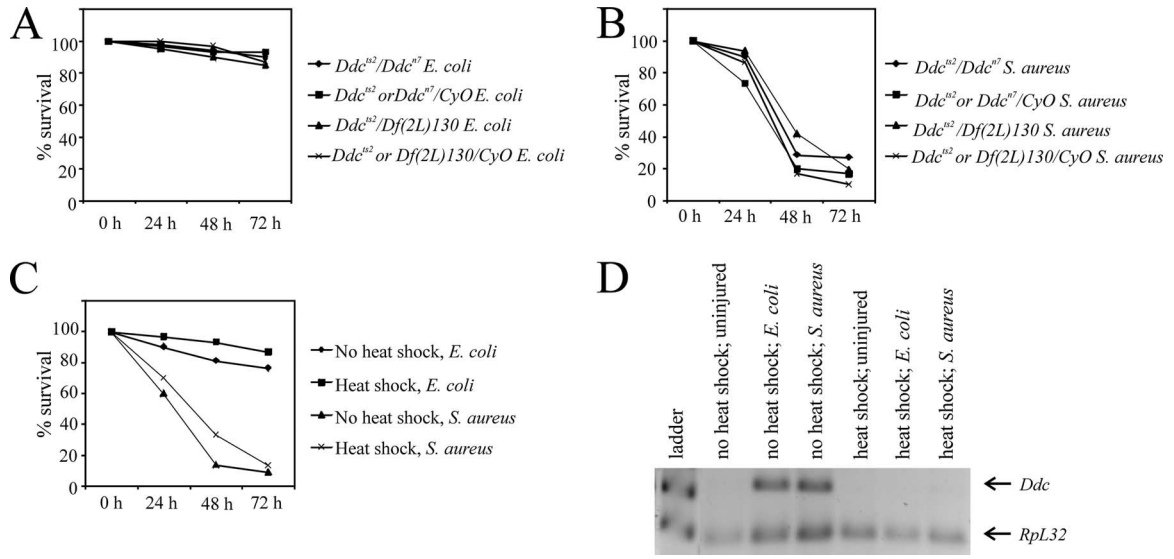

FIG. 4. Reduction of DDC activity does not affect survival following septic injury. Survival of *Ddc* mutant flies and *Ddc*-RNAi-expressing flies after infection with *E. coli* or *S. aureus* was tracked for 3 days. (A and B) Survival of the straight-winged heteroallelic *Ddc* mutant combinations was compared to curly-winged wild-type sibling survival following septic injury with *E. coli* or *S. aureus*. (C) Survival of *hs*-Gal4; P[*Ddc*-RNAi]pWIZ flies following heat shock (1 h at 37°C, 1 h of recovery) and no heat shock (control) after septic injury with *E. coli* or *S. aureus*. (D) Semiquantitative RT-PCR shows that *Ddc* transcripts are degraded in flies following heat shock induction of the *Ddc*-RNAi construct, but *Ddc* transcripts are detected in control (no heat shock) flies infected with *E. coli* and *S. aureus*.

epidermis is required for survival in the *Drosophila* immune response, we infected Ddc^{ts2}/Ddc^{n7} and $Ddc^{ts2}/Df(2L)130$ mutants with *E. coli* and *S. aureus* and tracked their survival for 3 days (Fig. 4). The Ddc^{n7} allele is a null mutation, and *Df(2L)130* is a deficiency of the locus; both are embryonic lethal. The Ddc^{ts2} mutation is a temperature-sensitive mutation, and $Ddc^{ts2}/Df(2L)130$ flies have 2 to 3% of the DDC activity detectible in wild-type flies (85). We find that there is no difference in the survival rates of Ddc^{ts2}/Ddc^{n7} (Fig. 4A) and $Ddc^{ts2}/Df(2L)130$ flies (Fig. 4B) compared to their wild-type siblings. Thus, we conclude that *Ddc* induction at 2 to 3% of normal has no effect on 3-day adult survival following infection with *E. coli* and *S. aureus*.

In an attempt to eliminate DDC activity completely, we employed RNA interference (RNAi) to degrade *Ddc* transcripts. To confirm that our *Ddc*-RNAi constructs were functional, we expressed the UAS-driven RNAi constructs under the control of an *Act5C*-Gal4 driver. We found that all of the *Act5C*-Gal4; P[*Ddc*-RNAi]pWIZ flies died at pupariation, a stage when a high level of DDC activity is required for the rapid tanning of the pupal case. However, it is unlikely that there is a complete loss of DDC activity in these flies, since *Ddc* null mutants are embryonic lethal (84). *Act5C*-Gal4; P[*Ddc*-RNAi]pSymp flies developed normally, suggesting that expression of this construct did not destroy all of the *Ddc* transcripts, so we eliminated these flies from further analysis. We infected flies of the genotype *hs*-Gal4; P[*Ddc*-RNAi]pWIZ with or without heat shock and found that there was no difference in lethalities between heat-shocked and control flies following infection (Fig. 4C). Degradation of *Ddc* transcripts following heat shock was confirmed by semiquantitative RT-PCR (Fig. 4D). It appears that reduction in DDC activity in these flies does not affect adult survival.

The time courses of *Ddc* transcription are different following gram-negative and gram-positive infections. While reducing DDC activity did not decrease the survival of infected flies, our results do show that *Ddc* transcription is induced in the immune response. We set out to determine the transcriptional profile of *Ddc* expression following infection with *E. coli* or *S. aureus*. Since *Ddc* transcript levels are high at the second to third moults and at pupariation during normal development (M. M. Davis, unpublished observation), we were unable to determine the time course of *Ddc* transcription following septic injury of third-instar larvae and chose, therefore, to analyze adults. Flies were septically injured with live (Fig. 5A) or heat-killed (Fig. 5B) bacteria and harvested at various time points. RT-PCR on RNA isolated from pools of adults collected at the indicated time points reveals that *Ddc* transcripts are first detectible about 1 h after infection with live (Fig. 5A, top gel) or heat-killed (Fig. 5B, top gel) *E. coli*. Levels peak within 3 h, remain high until 6 h after infection, and drop thereafter. The profile of *Ddc* transcription is different in response to septic injury with *S. aureus* (Fig. 5A, bottom gel). In this case, *Ddc* transcripts are first detected 2 h after infection; levels peak by 9 h and drop slowly thereafter. Similar to infection with live *S. aureus*, flies septically injured with heat-killed *S. aureus* (Fig. 5B, bottom gel) induce *Ddc* transcription, and transcripts are detectible 3 and 6 h after infection. However, *Ddc* transcripts are not detectible 9 and 12 h after infection with heat-killed *S. aureus*. Thus, a sustained response to *S. aureus* requires continued infection by live bacteria.

A conserved AP-1 binding site is necessary to induce *Ddc* transcription. To determine which regulatory sequence was responsible for *Ddc* induction in the immune response, we created *Ddc*-GFP reporter constructs containing various amounts of the *Ddc* 5′ regulatory region (Fig. 1). Cuticular

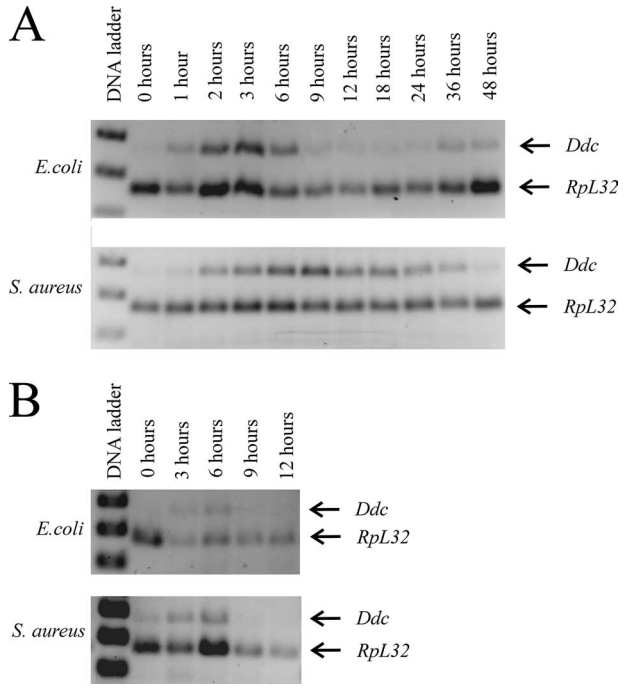

FIG. 5. Induction of *Ddc* transcription in adults occurs shortly after septic injury with live (A) or heat-killed (B) *E. coli* or *S. aureus*.

autofluorescence made confocal images of our epidermal preparations nearly impossible to analyze, so we employed semiquantitative RT-PCR to examine the expression of the reporter constructs. First, we infected flies bearing the constructs that contain the large deletions, P[*Ddc*-GFP]SH, P[*Ddc*-GFP]BH, and P[*Ddc*-GFP]EH, and compared reporter gene induction in these flies to expression in P[*Ddc*-GFP]PH-bearing flies (Fig. 6A, top gel). For all *Ddc*-GFP constructs, we examined expression following infection in a minimum of three independent transgenic lines. We find that the reporter gene is induced normally following both *E. coli* and *S. aureus* infection in flies bearing the P[*Ddc*-GFP]PH, P[*Ddc*-GFP]SH, and P[*Ddc*-GFP]BH constructs. However, GFP transcription is not induced in flies bearing the P[*Ddc*-GFP]EH construct. This suggests that the sequence necessary for *Ddc* induction is located between the BsmI and EcoRI restriction sites (Fig. 1). To confirm this, we infected a transgenic line bearing a construct, P[*Ddc*-GFP]PHΔBE, that deleted only this region (Fig. 1), and found that the reporter gene could not be induced following septic injury with *E. coli* or *S. aureus*. For each construct-bearing line, the wild-type copy of the endogenous *Ddc* gene was induced (Fig. 6A, bottom gel), showing that the immune response was triggered normally. To further narrow down the region responsible for *Ddc* induction, we created transgenic flies bearing reporter constructs that contained a deletion of part of the region between the BsmI and EcoRI sites. These were called P[*Ddc*-GFP]ΔBE-1, P[*Ddc*-GFP]ΔBE-2, and P[*Ddc*-GFP]ΔBE-3 (Fig. 1). We find that reporter gene transcription is immune inducible in flies bearing P[*Ddc*-GFP]ΔBE-1 and P[*Ddc*-GFP]ΔBE-2, but not in flies carrying P[*Ddc*-GFP]PHΔBE-3 (Fig. 6B, top gel). We conclude that a sequence contained within the 302-bp region, missing in P[*Ddc*-GFP]PHΔBE-3, is necessary for the induction of *Ddc* transcription in the innate immune response.

We aligned this 302-bp region (using the BLAT program; http://genome.ucsc.edu/) (37) with nine other sequenced *Drosophila* genomes. The sequence was also scanned for the presence of binding sites for known transcription factors found in the database TESS (72). This analysis revealed two conserved binding sites that could be involved in the *Drosophila* innate immune response (Fig. 7). These sites include a 7-bp AP-1 binding site and an 8-bp NF-κB binding site. The NF-κB binding site most closely resembles the recently defined Rel-specific binding site, not the Dorsal/DIF-specific sequence (11).

To determine if either of these sites was necessary for *Ddc* transcriptional induction in the innate immune response, we used site-directed mutagenesis (see Materials and Methods and see Table S2 in the supplemental material) to mutate 5 bases within each site to create P[*Ddc*-GFP]PHmutNFκB and P[*Ddc*-GFP]PHmutAP-1 (Fig. 1). When transgenic flies bearing the P[*Ddc*-GFP]PHmutNFκB construct were infected with either *E. coli* or *S. aureus*, reporter gene transcription was induced (Fig. 6B, top gel). This suggests that the NF-κB site plays no role in *Ddc* immune induction. The importance of an intact AP-1 site is apparent from the lack of reporter gene transcription in P[*Ddc*-GFP]PHmutAP-1 flies following infection with either bacterium. In both cases, septic injury induced transcription of the endogenous *Ddc* gene normally in reporter-bearing flies (Fig. 6B, bottom gel). An intact AP-1 site was also necessary for larval transcriptional induction (data not shown).

AP-1 can bind its putative site in vitro. Loss of reporter gene induction in P[*Ddc*-GFP]PHmutAP-1 suggests that the JNK-activated transcription factor complex, AP-1, may be involved in the activation of *Ddc* transcription in the innate immune response. To determine if AP-1 could bind its putative binding site upstream of *Ddc*, we employed an electrophoretic mobility shift assay (see Fig. S2 in the supplemental material). We find that the JUN/FOS heterodimer can bind the consensus AP-1 binding site, while neither subunit can independently bind the site. Retardation of the probe is eliminated by addition of a cold competitor and is absent when the probe is mutated, demonstrating the specificity of the binding reaction.

Neither Toll, IMD, nor JNK signaling is involved in *Ddc* immune induction. To determine if the AP-1 binding site was a target of JNK signaling, in vivo, we employed the bipartite GAL4/UAS system to express wild-type and dominant-negative forms of *Drosophila* MAPKK, HEP, and the JNK BSK, as well as JUN and FOS under the control of a heat shock promoter. In each case, we infected organisms that had been heat shocked for 1 h and then allowed to recover for 1 h prior to infection. Control organisms were not heat shocked. Expression of *hep* (UAS-*hep*), *bsk* (UAS-*bsk*), *fos* (UAS-*fra*), or *jun* (UAS-*jra*) did not precociously induce *Ddc* transcription in the absence of infection, although *Ddc* was immune inducible in these flies (see Fig. S3 in the supplemental material). Furthermore, expression of a constitutively active HEP (UAS-*hepAct*) did not precociously induce *Ddc* transcription (see Fig. S3A in the supplemental material). Finally, expression of dominant-negative forms of *bsk* (UAS-*bskDN*), *jun* (UAS-*junDN*), or *fos* (UAS-*fraDN*) did not eliminate immune induction of *Ddc* transcription (see Fig. S3B, C, and D in the supplemental material).

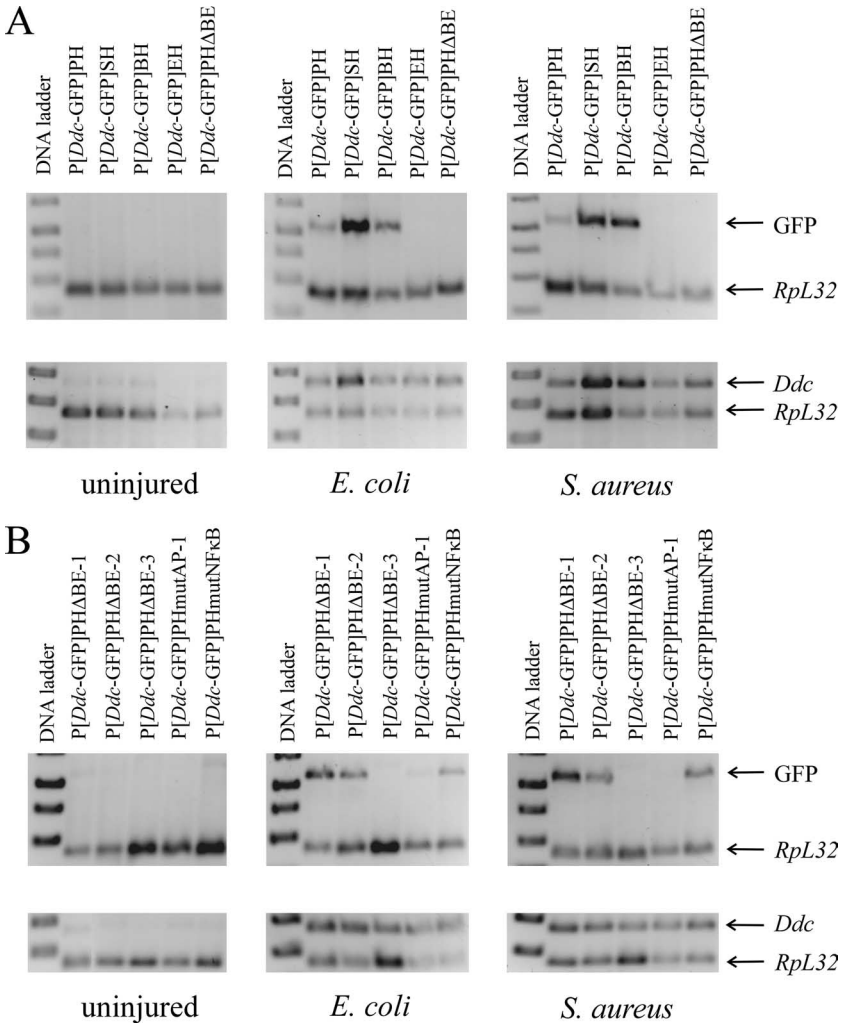

FIG. 6. Mapping the region upstream of *Ddc* that is required for immune inducibility. Semiquantitative RT-PCR of *Ddc*-GFP and endogenous *Ddc* transcripts was carried out on RNA isolated from adult flies bearing GFP reporter genes. *Ddc*-GFP or *Ddc* transcripts were co-reverse transcribed and PCR amplified with an *RpL32* loading control. (A) Deletions localizing the response element to the region from BsmI to EcoRI (Fig. 1). (B) The region from BsmI to EcoRI contains an AP-1 site that is necessary for immune inducibility of *Ddc*.

Ectopic expression of these JNK pathway components failed to indicate a role for JNK signaling in the *Ddc* immune response. Consistent with this observation, *Ddc* was shown not to be a JNK-inducible gene (9). However, since a conserved AP-1 binding site is necessary for *Ddc* immune induction, we thought it advisable to assess the effects of a JNK loss-of-function mutant on *Ddc* inducibility. *Tak1* mutant flies, in which the mutation eliminates both IMD and JNK signaling (73, 80), activate immune transcription of *Ddc* normally (Fig. 8A). This experiment provides strong evidence that the JNK pathway and its transcription factor complex, AP-1, are not involved in the induction of *Ddc* in the innate immune response. The identity of the transcription factor that acts through the AP-1 consensus site to effect *Ddc* induction remains unknown.

```
D. melanogaster   tg-tttcgagtgactcataat----tggGGGATTCCtgacg
D. simulans       tg-tttcgagtgactcatgtt-----ggGGGATTCCtgtcg
D. sechellia      tg-tttcgagtgactcatgat-----ggGGGATTCCtgtcg
D. yakuba         tg-tttcgagtgactcatgat-----ggGGGATTCCcgacg
D. erecta         tg-ttacgagtgactcatgat-----ggGGGATTCCtgacg
D. ananassae      tg-gattttgtgactcatgat-----ggGGGATTCCtg---
D. pseudoobscura  gg-tgtgacgtgactcatgac-----ggGGGATTCCtg---
D. persimilis     gg-tgtgacgtgactcatgac-----ggGGGATTCCtg---
D. willistoni     tgaccacttgtgattcatcacc-tg-tgGGGATTCCt----
D. grimshawi      tg-caacatgtgactcataagcgctgggGGAATTCTt----
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FIG. 7. Conserved AP-1 and NF-κB sites lie within the region deleted in P[*Ddc*-GFP]BE-3. Alignment of a portion of the *Ddc* region deleted in P[*Ddc*-GFP]BE-3 from 10 different *Drosophila* species reveals a conserved consensus AP-1 (underlined and bold) and a consensus NF-κB site (capital letters and bold) embedded in a less-conserved area. Deviations from the *D. melanogaster* sequence are italicized.

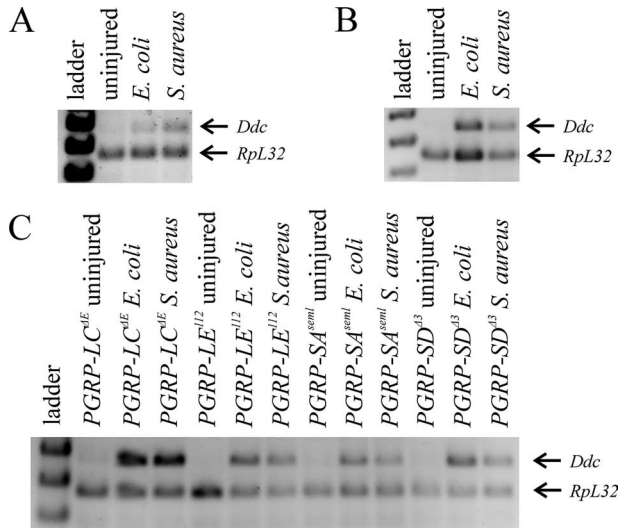

FIG. 8. Immune inducibility of *Ddc* is unaffected in JNK, ERK, and PGRP loss-of-function mutants. *Ddc* RT-PCR on RNA obtained from uninjured and septically injured *Tak1* (A), rl^1 (B), or PGRP (C) mutant flies.

We also infected mutants in the receptors responsible for detecting bacterial infection, the peptidoglycan recognition proteins (PGRPs). PGRP-LC and PGRP-LE are the major activators of the IMD pathway (25, 78), while PGRP-SA and PGRP-SD are responsible for induction of the Toll pathway (7, 56). We find that *Ddc* transcription is induced normally in $PGRP\text{-}LC^{\Delta E}$, $PGRP\text{-}LE^{112}$, $PGRP\text{-}SA^{seml}$, and $PGRP\text{-}SD^{\Delta 3}$ mutants following *E. coli* and *S. aureus* infection (Fig. 8C). We also infected mutants of additional components of the IMD pathway. These included $ird5^{KG08072}$ and $ird5^{EY02434}$, both of which have a P-element insertion in the first exon of the *ird5* gene, which encodes the β subunit of the IKK complex (48, 67, 74). This complex is essential for IMD signaling downstream of TAK1. In addition, we infected flies bearing a P-element insertion in the gene encoding the IMD-induced NF-κB transcription factor, Relish, $Rel^{EY08061}$. *Ddc* transcription was immune inducible in these each of these mutants (data not shown). Genetic data likewise fail to indicate a role for Toll signaling in *Ddc* immune induction. The Tl^3 gain-of-function mutant, which constitutively induces AMP expression in the absence of infection (45), did not precociously induce *Ddc* transcription (data not shown). These data provide strong evidence that the NF-κB site, although perfectly conserved, is not required for *Ddc* induction following septic injury.

The p38c MAPK pathway is responsible for *Ddc* induction. We have provided evidence that the major immune signaling pathways are dispensable for *Ddc* immune induction. Two other MAPK cascades have been implicated in *Drosophila* immunity and wound healing. The first, the ERK pathway, was shown by Mace et al. (49) to be necessary for robust induction of *Ddc* in the embryonic wound response. The *Drosophila* ERK is encoded by the gene *rolled* (*rl*) (6). We infected the hypomorphic rl^1 mutant with *E. coli* and *S. aureus* and found that *Ddc* was induced normally (Fig. 8B).

The second, the p38 MAPK pathway, has been implicated in the attenuation of the immune response (27). There are two p38 MAPKs that have been described in *Drosophila*, *p38a* and *p38b*. In addition, there is a third p38 gene, termed *p38c* (CG33338), which has been annotated in FlyBase. The *p38c* gene is located immediately downstream of *p38a* and likely arose by gene duplication (G. Manning, personal communication). Although *p38c* shares 61% DNA sequence identity to *p38a*, there is some doubt that it, in fact, encodes a functional p38 MAPK. The *p38c* gene has a mutation that changes the TGY domain that is characteristic of p38 MAPKs (51) to a TDH (C. R. Craig, personal communication), thereby preventing this motif from serving as a substrate for dual phosphorylation. Secondly, it has 2 amino acid (aa) substitutions within the 8-aa catalytic domain that could prevent the active site from functioning. However, 4 expressed sequence tags (ESTs) have been identified in a cDNA library obtained from the larval fat body of infected organisms (J. Carlson, personal communication). Furthermore, the p38c MAPK protein coding sequence is conserved, suggesting that *p38c* is not a pseudogene, and may, in fact, be functional (G. Manning, personal communication).

The $Mpk2^1$ null allele of *p38a* (13), along with P-element insertions upstream of *p38b* ($p38b^{KG01337}$ and $p38b^{KG02737}$) and within *p38c* ($p38c^{KG05834}$), was obtained, and flies were infected with *E. coli* or *S. aureus* (Fig. 9A). We find that *Ddc* transcription is induced normally in $Mpk2^1$, $p38b^{KG01337}$ (Fig. 9A), and $p38b^{KG02737}$ (data not shown) flies, but is not induced in $p38c^{KG05834}$ flies (Fig. 9A). The $p38c^{KG05834}$ allele contains an insertion of a P-element, in the center of the coding sequence, which most likely disrupts p38c function. However, this insertion is annotated in FlyBase as affecting *p38a* function. We performed RT-PCR for both *p38a* and *p38c* transcripts on RNA obtained from $Mpk2^1$ and $p38c^{KG05834}$ mutants to determine the nature of the $p38c^{KG05834}$ mutation. We find that *p38a* is transcribed in $p38c^{KG05834}$ flies but not $Mpk2^1$ flies (Fig. 9B), and the *p38c* transcript is detectible in $Mpk2^1$ mutants, but not in $p38c^{KG05834}$ flies (Fig. 9C). Thus, we conclude that the insertion in $p38c^{KG05834}$ affects the expression of *p38c* and not *p38a* in these flies.

To confirm a role for p38c MAPK in the induction of *Ddc* transcription in the immune response, we undertook a P-element excision scheme to recover additional mutants of this gene. The P-element is inserted into the coding sequence of *p38c*, and we expected that most excisions would generate new mutants of *p38c*, since P-element excisions often leave remnants following transposition (24, 76, 77). Homozygous $p38c^{KG05834}$ female flies (but not males) are sterile (M. Davis, unpublished observation). Since P{SUPor-P} contains a mini-$white^+$ gene, we were able to detect P-element excisions by appearance of a white-eyed phenotype. We obtained 91 P-element excisions. In an attempt to create homozygous stocks, we found that 6 were homozygous lethal, 12 were homozygous viable and fertile, and 73 were homozygous viable but infertile. We assumed that the six lines with mutations that were homozygous lethal contained larger deletions that resulted in the loss of nearby essential genes and eliminated these lines from further analysis. Cloning and sequencing of the P-element excision region of *p38c* from several lines that yielded homozygous progeny revealed that those lines that were female fertile contained precise P-element excisions, which restored the correct reading frame of *p38c*. In contrast, those lines that dis-

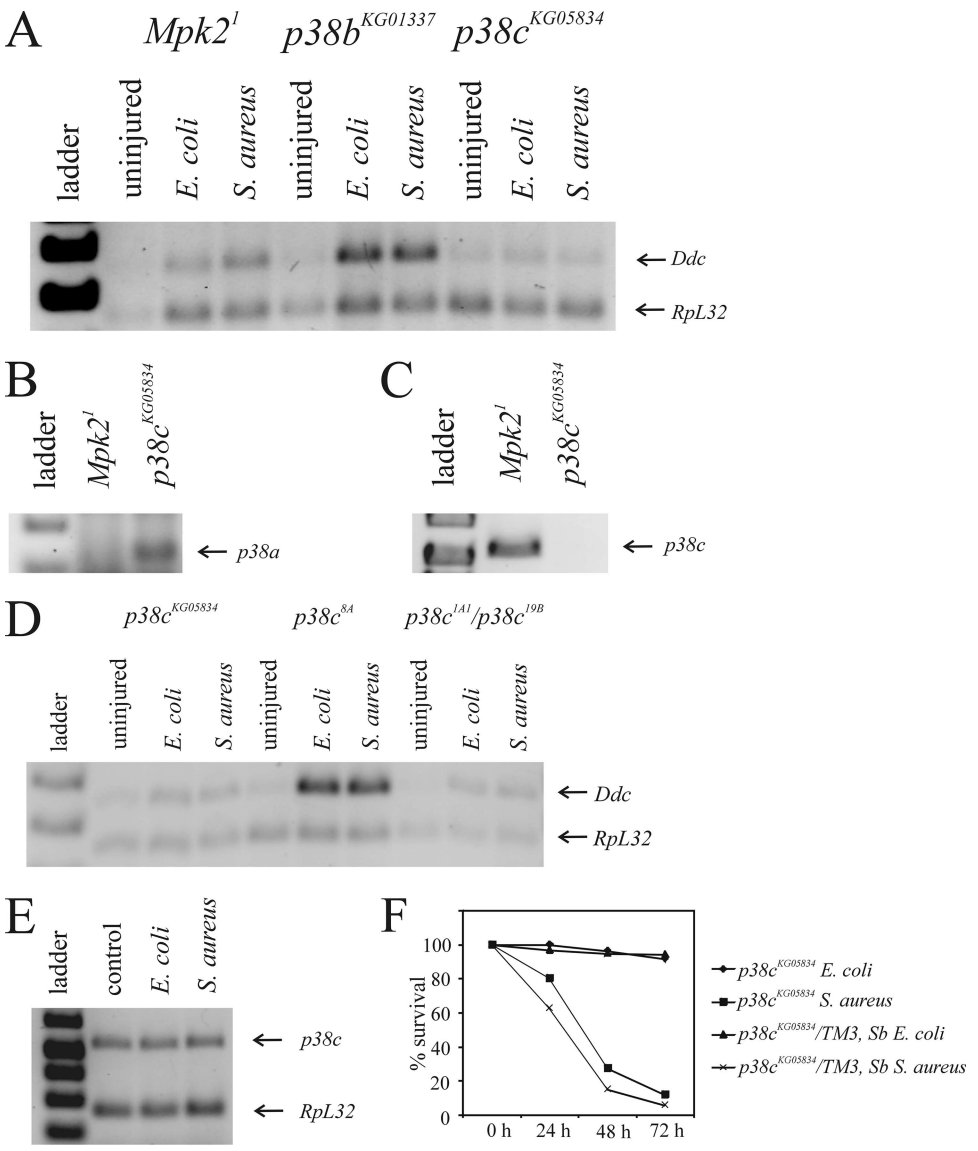

FIG. 9. p38c MAPK is required for immune induction of *Ddc*. (A) *Ddc* RT-PCR on RNA obtained from uninjured and infected $Mpk2^1$, $p38b^{KG01337}$, and $p38c^{KG05834}$ adult flies. (B) *p38a* transcript analysis in $Mpk2^1$ and $p38c^{KG05834}$ mutant flies. (C) *p38c* transcript analysis in $Mpk2^1$ and $p38c^{KG05834}$ mutant flies. (D) *Ddc* RT-PCR on RNA obtained from uninjured and infected $p38c^{KG05834}$, $p38c^{8A}$ (revertant of $p38c^{KG05834}$), and $p38c^{1A1}/p38c^{19B}$. (E) *p38c* transcription is not immune inducible. (F) Survival of $p38c^{KG05834}$homozygous flies and their $p38c^{KG05834}/TM3, Sb$ wild-type siblings following septic injury with *E. coli* and *S. aureus*.

played female infertility contained a mutation within *p38c*. Three revertant lines, $p38c^{2A}$, $p38c^{4C}$, and $p38c^{8A}$, were kept for further analysis. Three mutant lines ($p38c^{1A1}$, $p38c^{7B1}$, and $p38c^{19B1}$) were confirmed by sequencing to contain insertions that caused frameshift mutations that led to premature stop codons and truncated proteins. The full-length p38c MAPK is 356 aa long. The $p38c^{1A1}$ allele contained a 36-bp insertion that resulted in a 234-aa protein, the $p38c^{7B1}$ allele contained a 20-bp insertion that resulted in a 232-aa protein, and the $p38c^{19B1}$ allele contained a 71-bp insertion that resulted in a 239-aa protein. All three *p38c* mutants were female sterile, but the homozygous males were fertile.

$p38c^{1A1}$, $p38c^{7B1}$, and $p38c^{19B1}$ all failed to induce *Ddc* following infection with *E. coli* or *S. aureus* (data not shown). However, *Ddc* transcription was induced in $p38c^{2A}$, $p38c^{4C}$ (data not shown), and $p38c^{8A}$ (Fig. 9D) flies following septic injury with *E. coli* and *S. aureus*. To eliminate the possibility of second-site effects, we also examined five heteroallelic combinations of *p38c*, each of which failed to induce *Ddc* transcription following infection with *E. coli* or *S. aureus* (Fig. 9D and data not shown). The $p38c^{1A1}/p38c^{19B}$ flies shown in Fig. 9D demonstrate a typical heteroallelic result.

To analyze *p38c* expression, we used semiquantitative RT-PCR to detect *p38c* transcripts in uninjured and septically injured adult flies (Fig. 9E). We find that *p38c* transcription is not immune inducible; rather, levels of *p38c* transcripts are similar in infected and uninfected organisms.

Finally, to determine whether p38c is required for survival of

bacterial infection, we assessed the survival of $p38c^{KG05834}$ flies following infection with *E. coli* or *S. aureus*. We find that there is no difference in the survival of $p38c^{KG05834}$ flies compared with $p38c^{KG05834}$/*TM3, Sb* control flies following *E. coli* or *S. aureus* infection (Fig. 9F).

DISCUSSION

Microarray analysis has shown that *Ddc* transcription is induced by infection with gram-negative and gram-positive bacteria (9, 17, 34). We have confirmed that *Ddc* (Fig. 2), but not *ple* (data not shown), transcription is induced following gram-negative (*E. coli*) or gram-positive (*S. aureus*) septic injury with a small needle. The lack of *ple* transcriptional induction is not surprising, since PO activity can provide Dopa for DDC, thereby making TH redundant. In addition, transcripts for the genes encoding dihydropteridine reductase and GTP cyclohydrolase I, which are responsible for production of the essential TH cofactor, tetrahydrobiopterin, are significantly upregulated following septic injury (9, 17, 34). This induction is likely sufficient to increase basal TH activity levels.

Aseptic wounding of larvae or adults does not lead to *Ddc* transcriptional induction (Fig. 2), unlike in embryos where DDC activity at the edge of the wound contributes to the formation of a melanin clot (49). Our data show that robust induction of *Ddc* transcription is not apparent until 2 h after infection (Fig. 5), long after the melanin clot has already formed at the wound site. The profile of *Ddc* transcription differs in response to *E. coli* and *S. aureus* infection (Fig. 5). This discrepancy can be attributed to the virulence of *S. aureus*, since infection with heat-killed *S. aureus* recapitulates the profile of *Ddc* transcriptional induction with live *E. coli* (Fig. 5B).

Induction of *Ddc* transcription following septic injury apparently occurs within the epidermis (Fig. 3). Although we do not see a change in infection-induced *Ddc* transcription in dom^{K08108} larvae (Fig. 3B), we cannot eliminate the possibility that the hemocytes also express *Ddc*. There is a possibility that our dissected epidermal tissues are contaminated with small amounts of these cells; however, we view the likelihood of their contributing to the *Ddc* response as slight. Induction of *Ddc* transcription in the epidermal tissues following infection (Fig. 3C), but not wounding (Fig. 2 and 3A), suggests a role for *Ddc* in the destruction of invading microorganisms. We find that mutations in *Ddc* or knockdown of *Ddc* expression by RNAi does not affect the ability of *Drosophila* to survive infection (Fig. 4). This suggests that the low level of DDC activity in these flies is sufficient for survival. However, the actions of enzymes such as PO in the epidermis may be able to compensate for the loss of DDC activity in the immune response. Since all null alleles of *Ddc* are embryonic lethal (84), it is impossible to determine whether DDC activity is essential for survival following infection.

In an effort to determine which *Ddc* promoter element is responsible for transcriptional induction during the innate immune response, we infected larvae and adults of fly stocks bearing various *Ddc*-GFP reporter constructs (Fig. 1 and 6). We confirmed that the immune response was mounted in these flies by showing that transcription from the endogenous *Ddc* gene was induced (Fig. 6A, bottom gels). Infection of flies bearing constructs revealed that a 302-bp sequence that was deleted in P[*Ddc*-GFP]PHΔBE-3 was necessary for *Ddc* induction (Fig. 6B). Alignment of the sequences from 10 *Drosophila* species for this 302-bp region (Fig. 7) revealed conserved binding sites for NF-κB and AP-1 transcription factors. Although the NF-κB site is highly conserved in the 10 *Drosophila* species (Fig. 7), this site is dispensable for immune induction of *Ddc* transcription (Fig. 6B).

The Toll and IMD pathways both activate the immune response through NF-κB transcription factors. Mutations in Toll ($PGRP\text{-}SA^{seml}$, $PGRP\text{-}SD^{\Delta 3}$, and Tl^{3}) and IMD ($PGRP\text{-}LC^{\Delta E}$, $PGRP\text{-}LE^{112,\ ird5KG08072}$, $ird5^{EY02434}$, and $Rel^{EY08061}$) pathway components had no effect on *Ddc* immune induction following *E. coli* or *S. aureus* infection (Fig. 8C and data not shown). This confirms that the NF-κB site is not necessary for *Ddc* immune induction in larvae and adults following bacterial infection. However, *Ddc* could be developmentally regulated by Toll signaling, particularly during embryogenesis when Toll signaling is very important for embryonic patterning (75) and the role of *Ddc* is not well understood.

Mutation of the consensus AP-1 site eliminated the immune induction of the *Ddc*-GFP reporter construct (Fig. 6B), suggesting that a transcription factor must bind this site to induce *Ddc* transcription. *Drosophila* AP-1 is a heterodimer composed of one JUN subunit and one FOS subunit (60). We demonstrated that AP-1 could bind its putative site within the *Ddc* promoter, but not a mutated site, and that binding could be eliminated by the addition of a specific competitor (see Fig. S2 in the supplemental material). AP-1 is activated by phosphorylation carried out by JNK (16). Using a *hs*-Gal4 driver, we induced ectopic expression of two kinases in the JNK pathway, HEP and BSK. Neither these nor a constitutively active HEP (UAS-*hepAct*) caused precocious induction of *Ddc* transcription in the absence of infection (see Fig. S3A and B in the supplemental material). In addition, induction of dominant-negative forms of HEP and BSK did not prevent induction of *Ddc* transcription following infection (see Fig. S3A and B in the supplemental material). Furthermore, heat shock induction of JUN and FOS expression did not activate *Ddc* transcription, and expression of a dominant-negative form of JUN or FOS did not eliminate *Ddc* immune induction (see Fig. S3C and D in the supplemental material). Most importantly, a mutant in *Tak1*, which lies at the branch point common to IMD and JNK signaling, fails to eliminate *Ddc* immune induction (Fig. 8A). Taken together, these results suggest that the JNK pathway is not responsible for *Ddc* transcriptional induction. Although AP-1 can bind the consensus binding site upstream of *Ddc* in vitro, our genetic data suggest that AP-1 does not induce *Ddc* transcription in vivo. Therefore, we conclude that a transcription factor other than AP-1 binds to this site to activate *Ddc* transcription.

We have provided extensive evidence that the JNK pathway and its transcription factor, AP-1, are not involved in the immune induction of *Ddc* transcription. The ERK pathway has been implicated in the induction of *Ddc* in the embryonic wound response, and treatment with a universal MAPK inhibitor reduces *Ddc* induction (49), suggesting that a MAPK is important for *Ddc* transcription. *Ddc* was immune inducible in the hypomorphic mutant of *Drosophila* ERK, rl^{1} (Fig. 8B), suggesting that the ERK pathway is not responsible for *Ddc*

immune induction. However, analysis of a null *rl* mutant would be necessary to confirm this observation.

The p38 MAPK pathway has been implicated in attenuation of the *Drosophila* immune response (27). A null allele of *p38a*, $Mpk2^{1}$, did not affect immune induction of *Ddc* transcription. No mutant for *p38b* has been identified, but *Ddc* transcription is induced normally in two lines bearing P-element insertions immediately upstream of the *p38b* gene. However, the insertions likely do not eliminate p38b MAPK function, and the creation of a null allele of *p38b* would be useful. Interestingly, the only mutant we identified that affected *Ddc* transcriptional induction was $p38c^{KG05834}$, which carries a P-element insertion in the coding sequence of an uncharacterized member of the p38 MAPK family, p38c (Fig. 9A). Furthermore, excision of this P-element restores immune inducibility of *Ddc* (Fig. 9D). We recovered three additional mutants in *p38c*, and *Ddc* immune induction is absent in these mutants, both as homozygotes (data not shown) or in heteroallelic combinations (Fig. 9D). The *p38c* gene is located immediately downstream of *p38a* and likely arose by gene duplication (G. Manning, personal communication). Four ESTs for *p38c* were detected in infected larval fat body cells (J. Carlson, personal communication). We have shown that this gene is expressed within epidermal tissues (data not shown), although its transcript is not induced in the immune response (Fig. 9E). Transcription of *p38c* is lost in $p38c^{KG05834}$ (Fig. 9B), and the lack of *p38c* expression correlates with the loss of *Ddc* immune induction (Fig. 9A and D). The recovery of additional *p38c* mutants that fail to induce *Ddc* following infection (Fig. 9D) and the restoration of *Ddc* immune induction in flies bearing precise P-element excisions further confirm that p38c MAPK is responsible for *Ddc* transcriptional induction.

Although we demonstrated that p38c MAPK is not required for survival of *E. coli* or *S. aureus* infection (Fig. 9F), this does not eliminate the possibility that it is required for survival of infection with *E. coli* and *S. aureus* under different conditions or infection with other bacterial species. A large repertoire of genes is induced following bacterial infection of *Drosophila*, primarily due to the activation of the Toll, IMD, and JNK pathways. This implies that a number of parallel defense responses are mounted simultaneously. Induction of the p38c MAPK pathway may only be critical to the survival of organisms under very specific circumstances, as was shown for the role of NF-κB-induced immunity in the gut epithelia (69). In this case, the NF-κB-induced immunity is only critical for survival of infections with reactive oxygen species-resistant bacteria that cannot be killed by the reactive oxygen species-dependent immunity that destroys the majority of gut-infecting organisms.

The identification of yet another signaling pathway that participates in the immune induction of gene transcription points to the evolutionary significance of innate immunity. The p38c MAPK pathway elicits its response through the activation of an unknown transcription factor, which acts through the conserved consensus AP-1 site upstream of the *Ddc* transcription start site. It has been suggested that p38c MAPK cannot be activated by phosphorylation by a MAPKK due to the mutation that converts the TGY dual-phosphorylation motif to TDH (C. R. Craig, personal communication). However, a clear role for p38c MAPK in *Ddc* immune induction has been demonstrated, although the mechanism of p38c activation is still to be elucidated. It is clear that p38c MAPK is not activated by signaling through PGRP-LC, -LE, -SA, or -SD, since *Ddc* is induced normally following infection of these mutants (Fig. 8C). However, the possibility exists that another PGRP is responsible for the detection of invading bacteria and signaling to p38c MAPK.

It has been suggested that the amino acid substitutions within the catalytic domain may render p38c MAPK nonfunctional as a kinase (C. R. Craig, personal communication). A clear role for another putative kinase, Tribbles, in regulating the *Drosophila* Cdc25 homolog, String, during morphogenesis has been shown, although researchers have failed to demonstrate any kinase activity of this protein or any of its homologs (26, 29, 52). Further work is required to ascertain the catalytic activity of p38c MAPK, although it is possible that this protein has a regulatory role in the absence of kinase activity.

Delineation of the other components of the p38c MAPK signaling pathway will aide in our understanding of this system. The MAPKKK, MEKK1, has been shown to be the major activator of peptidoglycan-induced p38 MAPK activation in *Drosophila* (88), although future research will determine if it is responsible for p38c MAPK activation in the immune response.

The importance of DDC to neuronal production of DA is well established. During development, DDC produces the DA that is required for melanin and sclerotin production (2, 83). Tanning of the pupal case at pupariation and the hardening and darkening of the adult cuticle following eclosion are the most obvious manifestations of DA metabolism. *Ddc* transcription is also induced in the *Drosophila* embryonic wound response, at the wound site, to produce a melanin plug (49). Melanotic encapsulation of the parasitic wasp egg also requires DDC (57). In addition to these well-established roles for DDC, here, we demonstrate a role for the activation of transcription of this gene in the *Drosophila* innate immune response to bacterial infection. The appearance of DDC in the epidermis will increase the production of DA. The DA is most likely metabolized to produce reactive quinones that are toxic to invading bacteria. Presumably, DA is metabolized by the enzymes responsible for the production of sclerotin since no melanin deposits are evident within the epidermis. Consistent with this prediction, the gene *black*, whose protein product is an enzyme involved in NBAD synthesis (30, 62, 82), is also upregulated in the *Drosophila* immune response (34). Future research will reveal the extent of the antimicrobial role of quinone production in the innate immune response.

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REFERENCES

1. **Abramoff, M. D., P. J. Magelhaes, and S. J. Ram.** 2004. Image processing with Image J. Biophotonics Int. **11:**36–42.
2. **Andersen, S. O., P. Hojrup, and P. Roepstorff.** 1995. Insect cuticular proteins. Insect Biochem. Mol. Biol. **25:**153–176.
3. **Barolo, S., L. A. Carver, and J. W. Posakony.** 2000. GFP and beta-galacto-

sidase transformation vectors for promoter/enhancer analysis in Drosophila. BioTechniques **29:**726–732.
4. **Bidla, G., M. S. Dushay, and U. Theopold.** 2007. Crystal cell rupture after injury in Drosophila requires the JNK pathway, small GTPases and the TNF homolog Eiger. J. Cell Sci. **120:**1209–1215.
5. **Bidla, G., M. Lindgren, U. Theopold, and M. S. Dushay.** 2005. Hemolymph coagulation and phenoloxidase in Drosophila larvae. Dev. Comp. Immunol. **29:**669–679.
6. **Biggs, W. H., III, K. H. Zavitz, B. Dickson, A. van der Straten, D. Brunner, E. Hafen, and S. L. Zipursky.** 1994. The Drosophila *rolled* locus encodes a MAP kinase required in the sevenless signal transduction pathway. EMBO J. **13:**1628–1635.
7. **Bischoff, V., C. Vignal, I. G. Boneca, T. Michel, J. A. Hoffmann, and J. Royet.** 2004. Function of the drosophila pattern-recognition receptor PGRP-SD in the detection of Gram-positive bacteria. Nat. Immunol. **5:**1175–1180.
8. **Bosch, M., F. Serras, E. Martin-Blanco, and J. Baguna.** 2005. JNK signaling pathway required for wound healing in regenerating Drosophila wing imaginal discs. Dev. Biol. **280:**73–86.
9. **Boutros, M., H. Agaisse, and N. Perrimon.** 2002. Sequential activation of signaling pathways during innate immune responses in Drosophila. Dev. Cell **3:**711–722.
10. **Braun, A., J. A. Hoffmann, and M. Meister.** 1998. Analysis of the Drosophila host defense in *domino* mutant larvae, which are devoid of hemocytes. Proc. Natl. Acad. Sci. USA **95:**14337–14342.
11. **Busse, M. S., C. P. Arnold, P. Towb, J. Katrivesis, and S. A. Wasserman.** 2007. A kappaB sequence code for pathway-specific innate immune responses. EMBO J. **26:**3826–3835.
12. **Corbo, J. C., and M. Levine.** 1996. Characterization of an immunodeficiency mutant in Drosophila. Mech. Dev. **55:**211–220.
13. **Craig, C. R., J. L. Fink, Y. Yagi, Y. T. Ip, and R. L. Cagan.** 2004. A Drosophila p38 orthologue is required for environmental stress responses. EMBO Rep. **5:**1058–1063.
14. **Davis, M. M., S. L. O'Keefe, D. A. Primrose, and R. B. Hodgetts.** 2007. A neuropeptide hormone cascade controls the precise onset of post-eclosion cuticular tanning in Drosophila melanogaster. Development **134:**4395–4404.
15. **Davis, M. M., P. Yang, L. Chen, S. L. O'Keefe, and R. B. Hodgetts.** 2007. The orphan nuclear receptor DHR38 influences transcription of the DOPA decarboxylase gene in epidermal and neural tissues of Drosophila melanogaster. Genome **50:**1049–1060.
16. **Davis, R. J.** 2000. Signal transduction by the JNK group of MAP kinases. Cell **103:**239–252.
17. **De Gregorio, E., P. T. Spellman, G. M. Rubin, and B. Lemaitre.** 2001. Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays. Proc. Natl. Acad. Sci. USA **98:**12590–12595.
18. **Delaney, J. R., S. Stoven, H. Uvell, K. V. Anderson, Y. Engstrom, and M. Mlodzik.** 2006. Cooperative control of Drosophila immune responses by the JNK and NF-kappaB signaling pathways. EMBO J. **25:**3068–3077.
19. **Dushay, M. S., B. Asling, and D. Hultmark.** 1996. Origins of immunity: Relish, a compound Rel-like gene in the antibacterial defense of Drosophila. Proc. Natl. Acad. Sci. USA **93:**10343–10347.
20. **Ferrandon, D., A. C. Jung, M. Criqui, B. Lemaitre, S. Uttenweiler-Joseph, L. Michaut, J. Reichhart, and J. A. Hoffmann.** 1998. A drosomycin-GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway. EMBO J. **17:**1217–1227.
21. **Galko, M. J., and M. A. Krasnow.** 2004. Cellular and genetic analysis of wound healing in Drosophila larvae. PLoS Biol. **2:**E239.
22. **Giordano, E., R. Rendina, I. Peluso, and M. Furia.** 2002. RNAi triggered by symmetrically transcribed transgenes in *Drosophila melanogaster*. Genetics **160:**637–648.
23. **Glise, B., H. Bourbon, and S. Noselli.** 1995. *hemipterous* encodes a novel Drosophila MAP kinase kinase, required for epithelial cell sheet movement. Cell **83:**451–461.
24. **Gloor, G. B., J. Moretti, J. Mouyal, and K. J. Keeler.** 2000. Distinct P-element excision products in somatic and germline cells of Drosophila melanogaster. Genetics **155:**1821–1830.
25. **Gottar, M., V. Gobert, T. Michel, M. Belvin, G. Duyk, J. A. Hoffmann, D. Ferrandon, and J. Royet.** 2002. The Drosophila immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. Nature **416:**640–644.
26. **Grosshans, J., and E. Wieschaus.** 2000. A genetic link between morphogenesis and cell division during formation of the ventral furrow in Drosophila. Cell **101:**523–531.
27. **Han, Z. S., H. Enslen, X. Hu, X. Meng, I.-H. Wu, T. Barrett, R. J. Davis, and Y. T. Ip.** 1998. A conserved p38 mitogen-activated protein kinase pathway regulates *Drosophila* immunity gene expression. Mol. Cell. Biol. **18:**3527–3539.
28. **Hedengren, M., B. Asling, M. S. Dushay, I. Ando, S. Ekengren, M. Wihlborg, and D. Hultmark.** 1999. Relish, a central factor in the control of humoral but not cellular immunity in Drosophila. Mol. Cell **4:**827–837.
29. **Hegedus, Z., A. Czibula, and E. Kiss-Toth.** 2007. Tribbles: a family of kinase-like proteins with potent signalling regulatory function. Cell. Signal. **19:**238–250.
30. **Hodgetts, R. B.** 1972. Biochemical characterization of mutants affecting the metabolism of β-alanine in Drosophila. J. Insect Physiol. **18:**937–947.
31. **Hoffmann, J. A., F. C. Kafatos, C. A. Janeway, and R. A. Ezekowitz.** 1999. Phylogenetic perspectives in innate immunity. Science **284:**1313–1318.
32. **Imler, J. L., and P. Bulet.** 2005. Antimicrobial peptides in Drosophila: structures, activities and gene regulation. Chem. Immunol. Allergy **86:**1–21.
33. **Ip, Y. T., M. Reach, Y. Engstrom, L. Kadalayil, H. Cai, S. Gonzalez-Crespo, K. Tatei, and M. Levine.** 1993. Dif, a dorsal-related gene that mediates an immune response in Drosophila. Cell **75:**753–763.
34. **Irving, P., L. Troxler, T. S. Heuer, M. Belvin, C. Kopczynski, J. M. Reichhart, J. A. Hoffmann, and C. Hetru.** 2001. A genome-wide analysis of immune responses in Drosophila. Proc. Natl. Acad. Sci. USA **98:**15119–15124.
35. **Kallio, J., A. Leinonen, J. Ulvila, S. Valanne, R. A. Ezekowitz, and M. Ramet.** 2005. Functional analysis of immune response genes in Drosophila identifies JNK pathway as a regulator of antimicrobial peptide gene expression in S2 cells. Microbes Infect. **7:**811–819.
36. **Karlsson, C., A. M. Korayem, C. Scherfer, O. Loseva, M. S. Dushay, and U. Theopold.** 2004. Proteomic analysis of the Drosophila larval hemolymph clot. J. Biol. Chem. **279:**52033–52041.
37. **Kent, W. J.** 2002. BLAT—the BLAST-like alignment tool. Genome Res. **12:**656–664.
38. **Kim, L. K., U. Y. Choi, H. S. Cho, J. S. Lee, W. B. Lee, J. Kim, K. Jeong, J. Shim, J. Kim-Ha, and Y. J. Kim.** 2007. Down-regulation of NF-kappaB target genes by the AP-1 and STAT complex during the innate immune response in Drosophila. PLoS Biol. **5:**e238.
39. **Kim, M. H., C. H. Joo, M. Y. Cho, T. H. Kwon, K. M. Lee, S. Natori, T. H. Lee, and B. L. Lee.** 2000. Bacterial-injection-induced syntheses of N-beta-alanyldopamine and Dopa decarboxylase in the hemolymph of coleopteran insect, Tenebrio molitor larvae. Eur. J. Biochem. **267:**2599–2608.
40. **Kockel, L., J. G. Homsy, and D. Bohmann.** 2001. Drosophila AP-1: lessons from an invertebrate. Oncogene **20:**2347–2364.
41. **Lazareva, A. A., G. Roman, W. Mattox, P. E. Hardin, and B. Dauwalder.** 2007. A role for the adult fat body in Drosophila male courtship behavior. PLoS Genet. **3:**e16.
42. **Lee, Y. S., and R. W. Carthew.** 2003. Making a better RNAi vector for Drosophila: use of intron spacers. Methods **30:**322–329.
43. **Lemaitre, B., and J. Hoffmann.** 2007. The host defense of *Drosophila melanogaster*. Annu. Rev. Immunol. **25:**697–743.
44. **Lemaitre, B., E. Kromer-Metzger, L. Michaut, E. Nicolas, M. Meister, P. Georgel, J. M. Reichhart, and J. A. Hoffmann.** 1995. A recessive mutation, immune deficiency (*imd*), defines two distinct control pathways in the Drosophila host defense. Proc. Natl. Acad. Sci. USA **92:**9465–9469.
45. **Lemaitre, B., E. Nicolas, L. Michaut, J. M. Reichhart, and J. A. Hoffmann.** 1996. The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. Cell **86:**973–983.
46. **Levashina, E. A., S. Ohresser, B. Lemaitre, and J. L. Imler.** 1998. Two distinct pathways can control expression of the gene encoding the Drosophila antimicrobial peptide metchnikowin. J. Mol. Biol. **278:**515–527.
47. **Lindsley, D. L., and G. G. Zimm.** 1992. The genome of *Drosophila melanogaster*, vol. 8. Academic Press, San Diego, CA.
48. **Lu, Y., L. P. Wu, and K. V. Anderson.** 2001. The antibacterial arm of the Drosophila innate immune response requires an IkappaB kinase. Genes Dev. **15:**104–110.
49. **Mace, K. A., J. C. Pearson, and W. McGinnis.** 2005. An epidermal barrier wound repair pathway in Drosophila is mediated by grainy head. Science **308:**381–385.
50. **Manfruelli, P., J. M. Reichhart, R. Steward, J. A. Hoffmann, and B. Lemaitre.** 1999. A mosaic analysis in Drosophila fat body cells of the control of antimicrobial peptide genes by the Rel proteins Dorsal and DIF. EMBO J. **18:**3380–3391.
51. **Martin-Blanco, E.** 2000. p38 MAPK signalling cascades: ancient roles and new functions. Bioessays **22:**637–645.
52. **Mata, J., S. Curado, A. Ephrussi, and P. Rorth.** 2000. Tribbles coordinates mitosis and morphogenesis in Drosophila by regulating string/CDC25 proteolysis. Cell **101:**511–522.
53. **Meister, M.** 2004. Blood cells of Drosophila: cell lineages and role in host defence. Curr. Opin. Immunol. **16:**10–15.
54. **Meister, M., and M. Lagueux.** 2003. Drosophila blood cells. Cell. Microbiol. **5:**573–580.
55. **Meng, X., B. S. Khanuja, and Y. T. Ip.** 1999. Toll receptor-mediated Drosophila immune response requires Dif, an NF-kappaB factor. Genes Dev. **13:**792–797.
56. **Michel, T., J. M. Reichhart, J. A. Hoffmann, and J. Royet.** 2001. Drosophila Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein. Nature **414:**756–759.
57. **Nappi, A. J., Y. Carton, and E. Vass.** 1992. Reduced cellular immune competence of a temperature-sensitive *Dopa decarboxylase* mutant strain of *Drosophila melanogaster* against the parasite *Leptopilina boulardi*. Comp. Biochem. Physiol. B Biochem. Mol. Biol. **101:**453–460.
58. **Nappi, A. J., and E. Ottaviani.** 2000. Cytotoxicity and cytotoxic molecules in invertebrates. Bioessays **22:**469–480.
59. **Perkins, K. K., A. Admon, N. Patel, and R. Tjian.** 1990. The Drosophila

Fos-related AP-1 protein is a developmentally regulated transcription factor. Genes Dev. **4:**822–834.
60. **Perkins, K. K., G. M. Dailey, and R. Tjian.** 1988. Novel Jun- and Fos-related proteins in Drosophila are functionally homologous to enhancer factor AP-1. EMBO J. **7:**4265–4273.
61. **Petersen, U. M., G. Bjorklund, Y. T. Ip, and Y. Engstrom.** 1995. The dorsal-related immunity factor, Dif, is a sequence-specific trans-activator of Drosophila Cecropin gene expression. EMBO J. **14:**3146–3158.
62. **Phillips, A. M., R. Smart, R. Strauss, B. Brembs, and L. E. Kelly.** 2005. The Drosophila *black* enigma: the molecular and behavioural characterization of the *black1* mutant allele. Gene **351:**131–142.
63. **Ramet, M., R. Lanot, D. Zachary, and P. Manfruelli.** 2002. JNK signaling pathway is required for efficient wound healing in Drosophila. Dev. Biol. **241:**145–156.
64. **Riesgo-Escovar, J. R., M. Jenni, A. Fritz, and E. Hafen.** 1996. The Drosophila Jun-N-terminal kinase is required for cell morphogenesis but not for DJun-dependent cell fate specification in the eye. Genes Dev. **10:**2759–2768.
65. **Rizki, T. M., R. M. Rizki, and R. A. Bellotti.** 1985. Genetics of a Drosophila phenoloxidase. Mol. Gen. Genet. **201:**7–13.
66. **Rutschmann, S., A. C. Jung, C. Hetru, J. M. Reichhart, J. A. Hoffmann, and D. Ferrandon.** 2000. The Rel protein DIF mediates the antifungal but not the antibacterial host defense in Drosophila. Immunity **12:**569–580.
67. **Rutschmann, S., A. C. Jung, R. Zhou, N. Silverman, J. A. Hoffmann, and D. Ferrandon.** 2000. Role of Drosophila IKK gamma in a toll-independent antibacterial immune response. Nat. Immunol. **1:**342–347.
68. **Rutschmann, S., A. Kilinc, and D. Ferrandon.** 2002. Cutting edge: the toll pathway is required for resistance to gram-positive bacterial infections in Drosophila. J. Immunol. **168:**1542–1546.
69. **Ryu, J. H., E. M. Ha, C. T. Oh, J. H. Seol, P. T. Brey, I. Jin, D. G. Lee, J. Kim, D. Lee, and W. J. Lee.** 2006. An essential complementary role of NF-kappaB pathway to microbicidal oxidants in Drosophila gut immunity. EMBO J. **25:**3693–3701.
70. **Schachter, J., M. M. Perez, and L. A. Quesada-Allue.** 2007. The role of N-beta-alanyldopamine synthase in the innate immune response of two insects. J. Insect Physiol. **53:**1188–1197.
71. **Scherfer, C., C. Karlsson, O. Loseva, G. Bidla, A. Goto, J. Havemann, M. S. Dushay, and U. Theopold.** 2004. Isolation and characterization of hemolymph clotting factors in *Drosophila melanogaster* by a pullout method. Curr. Biol. **14:**625–629.
72. **Schug, J.** 2003. Using TESS to predict transcription factor binding sites in DNA sequence, chapter 2.6. *In* A. D. Baxevanis (ed.), Current protocols in bioinformatics. J. Wiley and Sons, Hoboken, NJ.
73. **Silverman, N., R. Zhou, R. L. Erlich, M. Hunter, E. Bernstein, D. Schneider, and T. Maniatis.** 2003. Immune activation of NF-kappaB and JNK requires Drosophila TAK1. J. Biol. Chem. **278:**48928–48934.
74. **Silverman, N., R. Zhou, S. Stoven, N. Pandey, D. Hultmark, and T. Maniatis.** 2000. A Drosophila IkappaB kinase complex required for Relish cleavage and antibacterial immunity. Genes Dev. **14:**2461–2471.
75. **Stathopoulos, A., and M. Levine.** 2002. Dorsal gradient networks in the Drosophila embryo. Dev. Biol. **246:**57–67.
76. **Staveley, B. E., T. R. Heslip, R. B. Hodgetts, and J. B. Bell.** 1995. Protected P-element termini suggest a role for inverted-repeat-binding protein in transposase-induced gap repair in Drosophila melanogaster. Genetics **139:** 1321–1329.
77. **Takasu-Ishikawa, E., M. Yoshihara, and Y. Hotta.** 1992. Extra sequences found at P element excision sites in Drosophila melanogaster. Mol. Gen. Genet. **232:**17–23.
78. **Takehana, A., T. Yano, S. Mita, A. Kotani, Y. Oshima, and S. Kurata.** 2004. Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in Drosophila immunity. EMBO J. **23:**4690–4700.
79. **Tzou, P., S. Ohresser, D. Ferrandon, M. Capovilla, J. M. Reichhart, B. Lemaitre, J. A. Hoffmann, and J. L. Imler.** 2000. Tissue-specific inducible expression of antimicrobial peptide genes in Drosophila surface epithelia. Immunity **13:**737–748.
80. **Vidal, S., R. S. Khush, F. Leulier, P. Tzou, M. Nakamura, and B. Lemaitre.** 2001. Mutations in the Drosophila *dTAK1* gene reveal a conserved function for MAPKKKs in the control of rel/NF-kappaB-dependent innate immune responses. Genes Dev. **15:**1900–1912.
81. **Walter, M. F., L. L. Zeineh, B. C. Black, W. E. McIvor, T. R. Wright, and H. Biessmann.** 1996. Catecholamine metabolism and *in vitro* induction of premature cuticle melanization in wild type and pigmentation mutants of *Drosophila melanogaster*. Arch. Insect Biochem. Physiol. **31:**219–233.
82. **Weber, J. P., R. J. Bolin, M. S. Hixon, and A. F. Sherald.** 1992. Beta-alanine transaminase activity in *black* and *suppressor of black* mutations of *Drosophila melanogaster*. Biochim. Biophys. Acta **1115:**181–186.
83. **Wright, T. R.** 1987. The genetics of biogenic amine metabolism, sclerotization, and melanization in *Drosophila melanogaster*. Adv. Genet. **24:**127–222.
84. **Wright, T. R., G. C. Bewley, and A. F. Sherald.** 1976. The genetics of dopa decarboxylase in *Drosophila melanogaster*. II. Isolation and characterization of dopa-decarboxylase-deficient mutants and their relationship to the α-methyl-dopa-hypersensitive mutants. Genetics **84:**287–310.
85. **Wright, T. R., R. Steward, K. W. Bentley, and P. N. Adler.** 1981. The genetics of Dopa decarboxylase in *Drosophila melanogaster*. III. Effect of a temperature sensitive Dopa decarboxylase deficient mutation of female fertility. Dev. Genet. **2:**223–235.
86. **Zettervall, C. J., I. Anderl, M. J. Williams, R. Palmer, E. Kurucz, I. Ando, and D. Hultmark.** 2004. A directed screen for genes involved in Drosophila blood cell activation. Proc. Natl. Acad. Sci. USA **101:**14192–14197.
87. **Zhang, K., J. R. Chaillet, L. A. Perkins, T. D. Halazonetis, and N. Perrimon.** 1990. Drosophila homolog of the mammalian jun oncogene is expressed during embryonic development and activates transcription in mammalian cells. Proc. Natl. Acad. Sci. USA **87:**6281–6285.
88. **Zhuang, Z. H., Y. Zhou, M. C. Yu, N. Silverman, and B. X. Ge.** 2006. Regulation of Drosophila p38 activation by specific MAP2 kinase and MAP3 kinase in response to different stimuli. Cell. Signal. **18:**441–448.