Dynamic Repositioning of Dorsal to Two Different κB Motifs Controls Its Autoregulation during Immune Response in Drosophila

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Autoregulation is one of the mechanisms of imparting feedback control on gene expression. Positive autoregulatory feedback results in induction of a gene, and negative feedback leads to its suppression. Here, we report an interesting mechanism of autoregulation operating on Drosophila Rel gene dorsal that can activate as well as repress its expression. Using biochemical and genetic approaches, we show that upon immune challenge Dorsal regulates its activation as well as repression by dynamically binding to two different κB motifs, κB1 (intronic κB) and κB2 (promoter κB), present in the dorsal gene. Although the κB1 motif functions as an enhancer, the κB2 motif acts as a transcriptional repressor. Interestingly, Dorsal binding to these two motifs is dynamic; immediately upon immune challenge, Dorsal binds to the κB1 leading to auto-activation, whereas at the terminal phase of the immune response, it is removed from the κB1 and repositioned at the κB2, resulting in its repression. Furthermore, we show that repression of Dorsal as well as its binding to the κB2 depends on the transcription factor AP1. Depletion of AP1 by RNA interference resulted in constitutive expression of Dorsal. In conclusion, this study suggests that during acute phase response dorsal is regulated by following two subcircuits: (i) DI-κB1 for activation and (ii) DI-AP1-κB2 for repression. These two subcircuits are temporally delineated and bring about overall regulation of dorsal during immune response. These results suggest the presence of a previously unknown mechanism of Dorsal autoregulation in immune-challenged Drosophila.

Insects have evolved simple yet multipronged strategies to defend themselves against microbial invasion. The mechanisms that regulate the different arms of insect immunity have been well investigated in Drosophila melanogaster (1). To combat microbial challenge, Drosophila relies on multiple defense reactions, which partly resemble the innate immune response of higher organisms (2–5). Such a conserved innate immune pathway suggests ancient origin of immune response during metazoan evolution. Because of this evolutionary conservation, Drosophila has emerged as a model for studying common innate immune mechanisms in animals (5, 6). For example, homologues of the cell surface receptor protein Toll of Drosophila, and its downstream signaling pathway, are present in mammals as well. Activation of the Toll pathway leads to synthesis of antimicrobial peptides (AMPs)3 in both insects and mammals (3, 7). The hallmark of Drosophila immune defense is the infection-induced synthesis and secretion of a battery of AMPs into the hemolymph by the fat body (8–10). These AMPs are the downstream effector molecules of the two immune pathways, namely Toll and Imd (3, 8–12).

To understand induction of AMP genes upon activation of Toll/Imd pathway, regulatory elements in their promoters were analyzed and mapped. The analysis revealed the presence and requirement of DNA motifs resembling the κB motifs of mammals for inducibility of immune genes upon infection in Drosophila (13). Later, three NF-κB/Rel-like proteins were also identified in Drosophila (14). Two of these, Dorsal (DI) and Dif, encoded by two clustered genes, are part of the Toll pathway signaling induced upon infection by Gram-positive bacteria or fungi (15, 16). Relish, the third member of this family, regulates induction of AMPs of the Imd pathway upon infection by Gram-negative bacteria (14, 17). Dif and Dorsal play redundant roles in regulating expression of drosomycin, a Toll pathway AMP gene, at the larval stage, whereas Dif alone mediates drosomycin expression in adults (15, 16). In Drosophila, activation of Toll upon microbial infection involves the recruitment of the adaptor protein Myd88, leading to the activation of the kinase Pelle and subsequent phosphorylation and degradation of Cactus, the cytoplasmic inhibitor of Dorsal and Dif, which brings about rapid nuclear translocation of these two transcription factors (17, 18). Dorsal also acts as a morphogen during embryonic development (19). In the early embryo, degradation of Cactus, upon developmental cues arising from activation of Toll, allows Dorsal to enter the nuclei along the dorso-ventral axis in a gradient. Formation of Dorsal gradient is important for the regulation of target gene expression involved in dorso-ventral patterning (19). However, the Toll signaling cascade controlling the AMP response differs from the dorso-ventral patterning pathway at the following two levels: (i) regulation by

3 The abbreviations used are: AMP, anti-microbial protein/peptide; TSS, transcription start site; LPS, lipopolysaccharide; PGN, peptidoglycan; hpi, hours post-infection; ChIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift assay; RNAi, RNA interference; PBS, phosphate-buffered saline; GFP, green fluorescent protein; dsRNA, double strand RNA.

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the serine proteases acting upstream of Spätzle in the signaling pathway, and (ii) use of Dif for immune response in the fat body, rather than Dorsal, which has a role in embryogenesis (8, 10, 17, 18).

Dorsal is a bifunctional transcription factor as it activates as well as suppresses transcription of target genes involved in embryonic development (20–22). For example, the twist enhancer region in Drosophila has multiple Dorsal-binding sites and is activated by Dorsal (23, 24), whereas Dorsal-binding sites in the zen promoter act as repressor elements (22). Point mutations in the Dorsal-binding motifs of the twist enhancer reduce ventral activation, whereas mutations in the Dorsal-binding sites in the zen silencer abolish ventral repression. These results suggest bi-functionality of Dorsal as a transcriptional activator as well as transcriptional repressor in vivo (20, 22, 25, 26).

Although there are many reports that emphasize regulation of Dorsal target genes, regulation of the doral (dl) gene itself has not been investigated thus far (27, 28). We are interested in understanding the molecular basis of sex-biased immune response in insects. While deciphering the molecular basis of the sex-biased immune response, we observed differential activation of Drosophila Rel proteins in the two sexes. We found that sex-differential activation of Rel proteins is modulated at different levels, including autoregulation (data not shown). In this study, we provide insights into the molecular mechanism underlying autoregulation of dl. We show that dl autoregulation is achieved by two different kb sites, a canonical kb motif (kbB) located in the first intron of dl and another functional but noncanonical kb motif (kbB′) present upstream of the transcription start site (TSS). We show that the kbB′ motif acts as an enhancer, whereas the kbB′′ motif is essential for the repression of dl at the termination of acute phase response. We found that Dorsal binding to the two motifs is dynamic and is temporally regulated. Immediately after immune challenge, Dorsal protein binds to the kbB′ motif, which results in immediate and strong expression of dl gene. However, later in acute phase response, Dorsal binding was located at the kbB′′ motif and not at the kbB′ motif. Further analysis suggested that dl expression at the onset of acute phase response was regulated by Dorsal alone; however, its repression at the end of acute phase response required interaction with another transcription factor AP1. Drosophila AP1 is a homodimer or heterodimer of Fra (Jun-related antigen) and Fra (Fos-related antigen). Here, we show that Drosophila AP1 acts as a co-repressor in dl regulation.

**EXPERIMENTAL PROCEDURES**

*Drosophila Stocks—w^{1118} flies were used as standard wild type strain. dl^{B} flies were obtained from Bloomington Stock Centre. All stocks were maintained and the experiments performed at 25 °C. Dsrgfp transgenic flies were provided by Bruno Lemaître, CNRS, France.

**Infection Experiments—**Third instar wandering stage Drosophila larvae, maintained at 25 °C, were infected with the Gram-positive bacterium *Micrococcus luteus* by pricking with a sharp needle dipped in a bacterial pellet with absorbance of ~100. Drosophila S2 cells were immune-challenged by adding 50 μg of lipopolysaccharide (LPS) (Sigma) and 50 μg of peptidoglycan (PGN) (Sigma) per well containing ~1 × 10^6 cells.

**Electrophoretic Mobility Shift Assay—**Embryonic nuclear extracts were prepared by homogenizing embryos (2–4-h-old) in extraction buffer (20 mM Hepes, pH 7.9, 5 mM MgCl2, 0.1 mM EGTA, 12.5% sucrose, 25% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride and protease inhibitor mixture) using a Dounce homogenizer, followed by centrifugation at 3000 × g for 15 min at 4 °C. The precipitated nuclei were suspended in 1 ml of extraction buffer. For EMSA, 100 ng each of different double-stranded oligonucleotide probes was labeled with 2 μl of [γ-32P]ATP (5 × 10^6 cpm) and 1 μl of polynucleotide kinase (10 units/μl) in 1 μl of PNK buffer (New England Biolabs) for 1 h at 37 °C. The labeled DNA was purified, and binding reaction was performed for 45 min at room temperature by mixing 1 ng of purified [32P]-labeled double-stranded synthetic oligonucleotide probe (4000 cpm/μl), 10 μl of nuclear extracts, and 300 ng of poly(dl-dC) in the presence of a protease inhibitor mixture (Sigma). Cold competition was performed by preincubating the extracts with a 50-fold excess of unlabeled oligonucleotide for 15 min at room temperature. For supershift experiment, anti-mouse Dorsal monoclonal antibody was added to the binding reaction for 30 min. The binding reaction was analyzed by electrophoresis on a 6% native polyacrylamide gel. The probe sequences are as follows: for kbB, ATGAGTCAGAAAAAACAAAGAAAAAACA; for mut-κB, ATGAGTCAGATAATATTCCAGAATAATCC, and for κB′, GGGAATTCGCGGATTCCCAGGGAATCC.

**Immunodepletion—**These experiments were performed essentially according to the protocols mentioned previously (29). Briefly, Dorsal was immunodepleted from the embryonic extract using anti-Dorsal monoclonal antibody obtained from Drosophila Studies Hybridoma Bank in a 100-μl final volume of buffer containing 30 mM Hepes-KOH, pH 7.4, 100 mM KOAc, 2 mM Mg(OAc)2, 2 mM dithiothreitol, and protease inhibitor mixture. Dorsal-depleted supernatant was collected and used immediately for coupled in vitro transcription and translation reaction.

**Coupled in Vitro Transcription and Translation—**Different luciferase constructs (1 μg each) were used for coupled in vitro transcription and translation. These plasmids were incubated with cell extracts prepared from LPS- and PGN-treated S2 cells. Additional supplements added in the reaction were RNase inhibitor, MgCl2, ATP, and amino acid mix as mentioned in Ref. 29. After adding all the components, the reaction was carried out for minimum of 2 h followed by Western analysis and/or luciferase assay.

**Western Blot Analysis—**Whole embryo extracts were prepared from 0- to 4-h-old dechorionated embryos in extraction buffer A (50 mM Tris, pH 7.5, 140 mM NaCl, 5 mM Mg(CH3COO)2, 0.05% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 mg of pepstatin A/ml, 10 mg of aprotinin/ml, and 1 mg of leupeptin/ml) at 40 °C.

Cell extracts containing 50 μg of protein were separated on a 10% SDS-polyacrylamide gel. The proteins were electrophoretically transferred to a Hybond-P membrane (Amersham Biosciences) using a Trans-blot cell (Bio-Rad), at 200 mA overnight at 4 °C. The blots were stained for total protein by Pon-
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Plasmid Constructs—The enhancer fragments were PCR-amplified from Drosophila genomic DNA with a 5′ primer containing a KpnI site and a 3′ primer containing an XhoI site and cloned into pG3L Basic vector. (Primer information is provided as supplemental Information 1.) Cloned inserts were verified by restriction digestion and sequencing. P3 is a full-length promoter construct (1.25-kb-long region upstream to TSS) with three κB motifs. We also generated a full-length enhancer construct with both upstream and downstream regulatory regions. The plasmid P3-Ex1-In1-Ex2 contained the exon1, Intron1, and part of exon2 apart from the 1.25-kb-long promoter region. To generate ΔκBκB plasmid, the NsiI recognition sequence surrounding the κBκB motif (AGAAAAACA) in the control P3-Ex1-In1-Ex2 plasmid was used to delete this motif by incubating with NsiI enzyme (New England Biolabs) followed by self-ligation. The same restriction site was used to insert the mutant κBκB motif harboring NsiI recognition sequence on either side of the mutant kappaκB motif sequence (AGAATACT) in P3-Ex1-In1-Ex2 plasmid that generated the plasmid κBκBmut. For motif swap experiment, the κBκB motif was cloned into the NsiI sites surrounding the κBκB motif. To generate κBκBmut plasmid, the mutant κBκB motif, GGGAAATAC, was used to replace the wild type κBκB (GGGAATTCC) by site-directed mutagenesis using QuickChange II site-directed mutagenesis kit (Stratagene) according to manufacturer’s protocol. The mutated nucleotides of the κBκB motif as part of the primer for site-directed mutagenesis are shown in boldface and underlined. To obtain ΔAP1 motif plasmid (AP1-del), the AP1 motif cluster in the control P3-Ex1-In1-Ex2 plasmid was deleted by restriction digestion with Mmelo only or both Mmelo and Nael. All the plasmids were purified using Qiagen columns. DNA was purified using Qiagen columns and later used in PCR. All PCRs were done at 62 °C for 30 cycles. The following primers were used for ChIP assay: primers used for amplifying κBκB motif, forward primer CAAA-GAAAATGGGAGGCCAG and the reverse primer AGAGA-GAGTGGCGCAAAGAGC. This primer pair amplifies 177-bp PCR product.

RNAi Knockdown—RNAi strategy was used to knockdown Drosophila AP1. Both AP1 components jra and fra of Drosophila were targeted as they can act either as homodimer or heterodimer. 219-bp region of the jra and 239 bp of fra open reading frames were amplified and cloned into TOPOII PCR cloning vector (Invitrogen) (supplemental Information 1). Double strand RNA was synthesized by in vitro transcription using T7 and SP6 RNA polymerases and annealed after ethanol precipitation. For RNAi, 1 × 106 Drosophila S2 cells were plated, washed with serum-free medium, and incubated in the same medium for 5–6 h. 5 μg of dsRNA, at 25 μg/ml concentration, was used for transfection. All transfections were done using FuGENE transfection reagent (Roche Applied Science) according to manufacturer’s protocol.

RESULTS

General Organization of Rel Promoters—Regulation of insect Rel genes is poorly understood. In silico analysis of regulatory sequences upstream of the transcription start sites of the three Drosophila Rel genes suggested the presence of putative binding sites for transcription factors like Dfd, Hb, Ftz, and BrCZ, but none of these is known to regulate immune response. However, one interesting prediction was the presence of κB motifs, which are known to be involved in regulation of immune response, in all three Rel gene promoters. GATA is another important regulator of immune response genes (30, 31). In a recent study, the existence and importance of a Rel-GATA module in the promoters of immune response genes, including AMP genes, was shown (28, 30). These GATA factors also impart tissue specificity and are known to modulate expression of AMP genes upon microbial infection (31). The presence of GATA motifs within 50 bp around the κB site, including their orientation with respect to the κB motif, was shown to be crucial for activation of AMP genes by Rel proteins upon immune challenge (28). The same Rel-GATA module was also found in the promoters of Dorsal target genes zen, rent, Ady, and fas3, which are expressed during embryonic stages, suggesting a common regulatory module in the Dorsal target genes (28). However, we did not find any Rel-GATA module either in the
vicinity or far away from the κB motif in the three Rel gene promoters. Thus, absence of the Rel-GATA module is a major difference between regulation of Rel genes and Rel target genes. We also found that the three Rel genes lacked TATA elements in their promoters. On the contrary, Rel target immune response genes have TATA elements as their basal promoters. Furthermore, relish and DIF promoters have a single canonical κB motif in their promoters; however, the same is not true for the dl gene, which has multiple κB motifs (supplemental Information 2 and supplemental Fig. 1). In light of this observation, we were interested to know how Rel genes are regulated. Here, we investigated transcriptional regulation of dl expression during acute phase immune response, both in vitro and in vivo.

dl Gene Is Autoregulated—Dorsal, which is a maternally expressed gene product, plays an important role in dorso-ventral patterning of the early embryo and also regulates expression of the antibacterial gene drosomycin in the bacteria-challenged larvae of Drosophila. However, induction of drosomycin, upon microbial infection, is compromised in dl mutant flies indicating absence of functional Dorsal (10, 15, 18). dl mutant is a loss-of-function (amorphic) mutation and shows a dorsalized embryo phenotype that ranges from D0 (completely dorsalized) to D3 (weakly dorsalized) (19). In the D0 phenotype, the cuticles of embryos lack ventral denticle belts, Filzkörper, and consist only of a tube of dorsal epidermis.

At the outset, we checked the status of Dorsal expression in dl mutant flies. Interestingly, in comparison with w1118 flies, the level of Dorsal in extracts prepared from dl embryos was extremely low or absent (Fig. 1A). Absence of the Dorsal protein in dl mutant could be due to the following: (i) instability of dorsal transcript or (ii) lack of Dorsal expression. We tested these two possibilities, in vitro, by luciferase reporter assay. The luciferase reporter plasmid used in this study consists of the dl regulatory region until the second exon of dl. Coupled in vitro transcription and translation of this reporter led to synthesis of luciferase with w1118 embryonic extract but not with extracts from the mutant dl embryos (Fig. 1B). This result is consistent with the result shown in Fig. 1A where a negligible amount of Dorsal was present in the dl extract (Fig. 1, A and B) suggesting that dl promoter is not inducible in dl embryonic extract. This result also implies that absence of Dorsal in Fig. 1A and luciferase in Fig. 1B, both under the regulation of dl promoter, is due to lack of expression of respective genes. Thus, emphasizing that reason for lack of Dorsal in dl embryo is dl deregulation and not mRNA instability. Lack of Dorsal in the dl mutant can be explained if we assume that Dorsal regulates expression of its own gene, i.e. dl gene is autoregulated (Fig. 1, A and B).

If Dorsal regulates its own expression, then depleting wild type Dorsal from w1118 embryonic extract should also result in no synthesis of luciferase. To test this hypothesis, we performed an immunodepletion experiment where wild type Dorsal was depleted from the w1118 embryonic extracts using anti-Dorsal monoclonal antibody. The Dorsal-depleted w1118 embryonic extract failed to synthesize luciferase upon coupled in vitro transcription and translation from the P3-Ex1-In1-Ex2:: luciferase reporter plasmid (Fig. 1C). Depletion of Dorsal, however, did not affect the coupled transcription and translation of GFP from an actin promoter-driven GFP construct, which was used as control plasmid, thus showing specificity of the immunodepletion reaction (Fig. 1C, lower band).

Identification of Autoregulatory Dorsal Enhancer Motif in dl Gene—Dorsal, like other transcription factors of the Rel family, binds to a consensus DNA sequence GGGRNNYYCC called the κB motif. All the Dorsal target genes have this motif in their regulatory regions. If Dorsal regulates its own expression, then
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One such κB motif should also be present in the dl promoter. We surveyed 5 kb upstream of TSS and 2 kb downstream to identify putative κB motifs in dl. The search revealed three putative κB motifs upstream and one downstream of TSS in the first intron (supplemental Information 2 and supplemental Fig. 1). Because the dl gene has multiple κB sites, we set out to identify the functional Dorsal-binding motif(s) by luciferase reporter assay. We generated the following two plasmids: (i) a 1.25-kb-long reporter construct (P3) that harbors the three κB motifs present upstream of TSS, and (ii) P3-Ex1-1n1-Ex2-luciferase plasmid, which also includes the intronic κB motif in addition to the three upstream κB motifs (Fig. 1D). Strong induction of luciferase was observed with the P3-Ex1-1n1-Ex2 construct compared with the P3 plasmid upon immune challenge to S2 cells. Lack of luciferase induction from the P3 plasmid suggested that none of the three κB motifs upstream of TSS might cause auto-activation of dl. Strong luciferase induction from P3-Ex1-1n1-Ex2 plasmid suggested that the functional Dorsal-binding motif was present downstream of TSS in the first intron (Fig. 1E). Next, the ability of Dorsal to interact physically with the intronic κB motif (GGGAATTC) named κB′, was also checked by gel shift assay (Fig. 2A). Furthermore, supershift with anti-Dorsal monoclonal antibody confirmed that the DNA-protein complex retarded in other lanes indeed included Dorsal (Fig. 2A, lane 3). The results confirmed the physical interaction of Dorsal with the regulatory region in the dl itself upon immune challenge.

ChIP Experiment Identifies Two Dorsal-binding Sites in dl Gene—ChIP was performed to confirm in vivo interaction of Dorsal with its own gene in immune-challenged S2 cells. ChIP was performed on the nuclear extracts prepared from PGN + LPS-treated S2 cells 15 h post-infection (hpi). Enrichment of the intronic κB (κB′) motif upon ChIP confirmed in vivo interaction of Dorsal with the regulatory motif κB′ (Fig. 2B). This is consistent with luciferase reporter assay and EMSA results (Figs. 1E and 2A). The three upstream κB motifs did not yield any PCR product when ChIP was performed with immune-challenged S2 cells 15 hpi. However, the second κB motif of the dl promoter, κB2, was enriched by PCR when ChIP was performed with the S2 cells 40 hpi (Fig. 2C). Because this was the only promoter κB motif that was precipitated upon ChIP, we named it κB″. These results suggested the presence of two functional Dorsal-binding κB motifs in the dl gene, one in the promoter, the κB′, and another one in the 1st intron, the κB″ (Fig. 2C). Lack of enrichment of the other two promoter κB motifs suggested that they were probably not functional (Fig. 2C).

Because ChIP experiments identified two Dorsal-binding motifs in the dl gene, we next tested how these two motifs regulated dl expression. Although the κB′ motif GGGAATTC is a typical κB motif, the κB″ motif AGAAAAACA is an atypical κB motif as it is significantly different from the consensus κB motif sequence GGGRNNYYCC. As the two motifs were enriched at different time points post-infection upon ChIP, we performed a time course of luciferase induction to elucidate the role of these two motifs in dl regulation. We checked the transcriptional activity of κB″ and κB′ individually in the same P3 promoter construct. For comparison, the κB″ motif in the P3 plasmid was replaced with the κB′ motif so that the two plasmids differed only in their Dorsal-binding sequences. Strong luciferase induction was observed with the P3-κB″ plasmid but not with the P3-κB′ plasmid under the same experimental conditions (Fig. 2D). In fact, luciferase induction with the P3-κB′ promoter was comparable with that of the full-length construct P3-Ex1-1n1-Ex2, suggesting the contribution of the κB′ motif in Dorsal activation (Figs. 1E and 2D). If the κB″ motif had no role in activation of dl, then what was the significance of its interaction with the Dorsal?

Dorsal Binding to κB′ and κB″ Motifs Is Dynamic and Temporally Regulated—To understand how the two Dorsal-binding motifs together regulate dl expression, we performed ChIP at different time points after immune activation in S2 cells. We found that immediately after bacterial challenge Dorsal was bound only to the κB′ motif, which explains Dorsal activation upon immune challenge (Fig. 3A). However, around 36 hpi, when the immune response had reached terminal stage, Dorsal binding was seen at the κB″ motif but not at the κB′ motif (Fig. 3A). This almost exclusive binding of Dorsal to two different κB motifs in the dl gene suggested that these two autoregulatory Dorsal-binding motifs independently regulate dl expression during the course of immune response. The critical feature of dl reg-

FIGURE 2. Identification and characterization of functional κB motifs in the dl gene. A, a dorsal-specific complex is retarded upon EMSA with κB motif as probe (marked by asterisk, lanes 4 and 5), which was supershifted with the Dorsal antibody (marked by arrowhead, lane 3). Lane 1, free probe; lane 2, cold homologous competition; lane 3, supershift with Dorsal antibody; lane 4, nuclear extract isolated from S2 cells 15 h after PGN + LPS treatment; lane 5, embryonic extract from 4-h-old w1118 embryo; lane 6, nonspecific competition. B, ChIP performed with anti-Dorsal antibody resulted in enrichment of κB motif. Chromatin for ChIP was precipitated from S2 cells 15 h after PGN + LPS treatment. C, ChIP was also performed for the upstream Dorsal-binding motifs. Of the three κB motifs, only κB2 of the dl promoter was enriched. None of the κB motifs were enriched in the dl′ mutant. D, luciferase assay suggested that the κB′ was inducible upon immune challenge but not the κB″. The schematics above the graphs show position and identity of κB motifs used in the dl promoter construct.
course of luciferase expression revealed a response similar to acute phase response, where initially there was a continuous increase in luciferase expression in the first 18 h, and by 40 h luciferase expression reached control levels (Fig. 4A). For the next experiment, we generated a \( \kappa B^\text{P}-\kappa B^\text{B} \) construct, by replacing the \( \kappa B^\text{B} \) motif with the \( \kappa B^\text{P} \), and found that this construct hardly showed any luciferase induction (Fig. 4B). In another experiment, the \( \kappa B^\text{P} \) motif in the control plasmid was replaced with the \( \kappa B^\text{B} \); the resultant luciferase-reporter construct, harboring two \( \kappa B^\text{P} \) motifs (\( \kappa B^\text{P} -\kappa B^\text{B} \)), caused strong luciferase induction and remained constitutively active (Fig. 4C). These results demonstrated the enhancer property of \( \kappa B^\text{B} \) motif in the regulation of \( dl \). Next, we swapped the two \( \kappa B \) motifs; the resultant new plasmid had \( \kappa B^\text{B} \) motif in the promoter and \( \kappa B^\text{P} \) motif in the first intron (\( \kappa B^\text{P} -\kappa B^\text{B} \) plasmid). We found that \( \kappa B^\text{B} -\kappa B^\text{P} \) construct was constitutively active, and there was no decrease in luciferase synthesis by 40 hpi as observed with the control \( \kappa B^\text{P} -\kappa B^\text{B} \) construct (Fig. 4, A and D).

These results highlight distinct roles played by the two autoregulatory \( \kappa B \) motifs in \( dl \) regulation. Although the \( \kappa B^\text{B} \) motif is responsible for initial activation of \( dl \), the \( \kappa B^\text{P} \) motif is probably required for \( dl \) repression (Fig. 4, A–D). The motif-swapping experiment clearly suggests that the \( \kappa B^\text{B} \) motif lacks enhancer activity, which also explains the lack of luciferase induction as seen in Figs. 1E, 2D, and 4B.

The motif-swapping experiment further revealed that it is not only the presence of the two \( \kappa B \) motifs but also their arrangement in the \( dl \) gene that is important for \( dl \) autoregulation during acute phase response. In other words, the \( \kappa B^\text{P} -\kappa B^\text{B} \) arrangement (where \( \kappa B^\text{P} \) is present upstream of TSS and \( \kappa B^\text{B} \) is present downstream) is required for initial activation and late repression of Dorsal in immune-challenged \( Drosophila \). However, it was not clear why in the initial phase of immune challenge Dorsal bound only to the \( \kappa B^\text{B} \) and not to the \( \kappa B^\text{P} \) motif considering that an abundant amount of Dorsal protein was available. We speculate that the mere presence of Dorsal protein in abundance is not sufficient for its binding to the \( \kappa B^\text{B} \) motif, and possibly time-dependent recruitment of Dorsal to \( \kappa B^\text{B} \) requires participation of other proteins and/or chromatin changes.

Dorsal Recruitment to \( \kappa B^\text{P} \) Motif Requires AP1 as Co-regulator—The motif swap experiment suggested the requirement of \( \kappa B^\text{P} -\kappa B^\text{B} \) arrangement for control of the expression dynamics of \( dl \), where \( \kappa B^\text{B} \) motif probably brings about the time-dependent repression of \( dl \), although initial induction is controlled by the
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FIGURE 4. Dorsal binding to the κBp and κBi motifs is distinctly regulated. A, luciferase expression construct mimicking the κBp-κBi organization in the dl gene shows rapid induction of luciferase expression followed by its repression by 40 hpi. B, replacing the κBp with the κBi resulted in κBp-κBi motif. This raised the intriguing question of how Dorsal recruitment to κBp motif is temporally regulated and also how it brings about repression of dl transcription. One possibility worth investigating was the role of one or more co-regulators, if any, in the positioning of Dorsal at the κBi motif, as it is known that Dorsal interacts with co-regulators for effecting transcriptional repression of target genes (20, 21, 26).

To find out if additional factors are involved in the recruitment of Dorsal to κBp or κBi motifs, we performed EMSA using κBp and κBi motifs as probes, which revealed retardation of complexes of different sizes (Fig. 5A). The protein complex recruited at the κBi motif was bigger in size compared with the complex retarded with the κBp motif, suggesting that one or more additional proteins were present in the Dorsal complex retarded with the κBi motif (Fig. 5A). Next, we investigated the following: (i) the identity of the co-regulatory protein(s) involved in Dorsal binding to the κBi motif, and (ii) whether the difference in protein-protein interaction can explain the difference in spatio-temporal regulation of dl by κBp and κBi motifs as seen in Figs. 3, A–C, and 4, A–D.

Transcriptional regulation of Dorsal target genes is sometimes modulated by other proteins; Groucho acts as co-repressor for Dorsal target genes along dorso-ventral axis (19), whereas GATA factors co-regulate AMP gene expression (30, 31). Because GATA-binding motifs are not present in the dl promoter (supplemental Information 2), and Groucho is not known to have a role in the immune response, we excluded these two proteins as candidates for modulation of dl expression. One potential candidate as co-regulator of Dorsal binding to the κBi motif that we came across after bioinformatic analysis of the dl promoter was AP1. A cluster of multiple AP1-binding sites is present just upstream of the κBi motif in the dl promoter (Fig. 5B and supplemental Information 2). To test if the AP1 and Dorsal-binding motifs interacted in cis, we generated different reporter plasmids with mutation in the two Dorsal-binding motifs and deletion of AP1-binding cluster (Fig. 5C). Because multiple AP1-binding motifs are clustered in the dl promoter, for functional analysis we deleted the AP1 cluster by restriction digestion in the full-length dl promoter, for functional analysis we deleted the AP1 cluster or mutated the AP1-binding motif in the dl promoter (Fig. 5D). To confirm the probable cross-talk between AP1 and Dorsal, we mutated the κBp motif in the P3-Ex1-In1-Ex2 plasmid to generate κBpmut plasmid. Luciferase expression from the κBpmut motif in the P3-Ex1-In1-Ex2 plasmid was constitutively active, whereas luciferase expression from the control P3-Ex1-In1-Ex2 plasmid underwent time-dependent repression (Fig. 5D). To confirm the probable cross-talk between AP1 and Dorsal, we mutated the κBp motif in the P3-Ex1-In1-Ex2 plasmid to generate κBpmut plasmid. Luciferase expression from the κBpmut motif suggests that both κBp and AP1-binding elements may be required for time-dependent dl organization that was hardly inducible. C, reporter construct where the κBp was replaced with the κBi and thus had two κBi motifs (κBi-κBi) and remained constitutively active. D, when the κBi and κBp motifs in the reporter κBp-κBi plasmid were swapped, the resultant κBp-κBi plasmid remained constitutively active. These results suggest that κBp-κBi organization (A) controls time-dependent activation followed by repression of the dl gene during acute phase response. The order and type of κB motifs in the promoter constructs are shown above the respective graphs.
repression during acute phase response (Fig. 5D). On the other hand, when the kBP was mutated (kBP-mut plasmid), no significant luciferase induction was observed at any time point, suggesting that the kBP motif is required for the dl activation (Fig. 5E). These results are consistent with motif swap experiments where replacing the kBP motif with the kB motif also resulted in constitutive expression of luciferase (Fig. 4D). Thus, our data confirm that the kBP is an enhancer motif and is required for the initial induction of dl (Figs. 5D and 3, A–D). On the other hand, the kB motif, occupied by Dorsal at the terminal stage of the acute phase response (Fig. 4, A–D), controls dl repression (Fig. 5, C and D). These data emphasize the presence of two temporally delineated Dorsal modules involved in dl autoregulation.

**Assembly of Dorsal-AP1 Complex at kBP Motif Leads to dl Repression**—To further dissect the role of AP1-Dorsal interaction in dl regulation, we took to the RNAi approach. The kBP-kBP reporter construct was co-transfected with AP1-dsRNA construct into S2 cells, and luciferase expression was quantitated at different time points after LPS + PGN treatment. We found that depletion of AP1 by RNAi resulted in loss of repression of dl with respect to control RNAi (Fig. 6A). However, when kBP-kBP plasmid was used for reporter assay in the presence of AP1-dsRNA, there was no repression of luciferase activity (Fig. 6B). These results further suggest the following: (i) kBP is an enhancer motif, and (ii) kBP functions as a repressor motif but only in the presence of AP1.

The RNAi data indicated that Dorsal-AP1 interaction may be responsible for repression of dl at the end of acute phase response. EMSA results have clearly shown retardation of a larger Dorsal-DNA complex with the kBP motif with respect to kBP motif (Fig. 5A). We speculated that the larger Dorsal-kBP complex probably also contained AP1 proteins apart from Dorsal. To test such a possibility, we performed EMSA using kBP as probe with whole and AP1-depleted nuclear extracts. A clear shift in gel retardation was seen between control (Fig. 6, A and B) and AP1-depleted nuclear extracts. A clear shift in gel retardation was seen between control (Fig. 6A) and AP1-depleted nuclear extracts (Fig. 6C, lanes 2–5). Depletion of AP1 resolves the control band (Fig. 6C, lane 1) into two (lanes 2–5) suggesting that AP1 interacts with the Dorsal-kBP complex (Fig. 6A and B). Depletion of AP1 by RNAi was also verified by EMSA, which showed progressive loss of AP1-specific band (Fig. 6D).

ChIP results and RNAi data together suggest that AP1 action is seen after 30 hpi (Figs. 6A, 2C, and 3, A and C). This may explain the recruitment of Dorsal to the kBP motif late in immune response but not in early stages. To follow the time-dependent assembly of the Dorsal-AP1 complex on the kBP...
motif, we performed competitive EMSA where the $\kappa B^P$ and $\kappa B^I$ motif probes were added together in equal concentration for the binding reaction. The mixture of the two probes was incubated with the nuclear extracts isolated at different time points from PGN/LPS-treated S2 cells so that the two complexes of Dorsal with $\kappa B^P$ and $\kappa B^I$ could be resolved in the same lane. Fig. 6E, lane 1 (where nuclear extract isolated at 15 hpi was used), shows a strong retardation of a smaller complex of Dorsal bound to $\kappa B^I$. However, with nuclear extracts isolated 24, 30, and 40 hpi (Fig. 6E, lanes 2–4), retardation of a higher size complex corresponding to Dorsal bound to $\kappa B^P$ is also seen. Results of competitive EMSA further emphasize that interaction of Dorsal with the two $\kappa B$ motifs is dynamic and temporally regulated.

Overall, our data suggest that the $\kappa B^I$ motif controls the induction of $dl$ seen in immune-challenged Drosophila, whereas the $\kappa B^P$ motif brings about the repression of $dl$ observed at the end of the acute phase response. The fact that Dorsal binding at these two motifs is time-dependent is suggestive of dynamic interaction of Dorsal with the two $\kappa B$ motifs. During the late phase of immune response, Dorsal is removed from the $\kappa B^I$ motif (Figs. 4, A–D, 5D, and 6D) and is repositioned at the $\kappa B^P$ motif. We have shown that the $\kappa B^P$ motif on its own is not a repressor motif (Figs. 2D and 3B), but it is the binding of Dorsal-AP1 complex to this motif that leads to repression of the downstream gene (Fig. 6, A–E). From the promoter swap experiments, we have shown that $dl$ expression requires both $\kappa B$ motifs in a $\kappa B^P$-$\kappa B^I$ orientation (Fig. 3, A–D).
Dorsal is a transcription factor and orchestrates many events, including embryonic development and immune response in *Drosophila* (32–35). We have shown that Dorsal acts during immune response via two subcircuits that dynamically interact with each other. The first subcircuit activates the *dl* gene with binding of Dorsal to the enhancer motif, *kBp*. This subcircuit gets established just after immune challenge and ensures supply of Dorsal during acute phase response. This positive feedback leads to accumulation of Dorsal protein during the post-infection period (Fig. 7A). But once the bacterial infection is cleared, the cell needs to come back to normal state, which requires shutting off the AMP genes, for which Dorsal availability has to be retrenched. At this point, as we have shown in this study, the second subcircuit involving AP1-*kBp* gets activated and causes removal of Dorsal from the *kBp* motif and its recruitment to the *kBp* motif.

Our data indicate that Dorsal bound to the *kBp* motif activates its own expression possibly by directly interacting with transcriptional machinery (Fig. 7). However, in later stage of acute phase response, binding of AP1 to *kBp* motif might cause localized chromatin changes facilitating its interaction with Dorsal but at the same time preventing interaction of Dorsal with transcriptional machinery to turn off *dl* transcription.

We believe that this repositioning of Dorsal is facilitated by localized chromatin changes in the *dl* gene region that lead to opening of the chromatin near the *kBp* motif in the promoter and contraction of chromatin near the *kB* motif in the first intron. As a result, the *kB* motif would become inaccessible to Dorsal. Simultaneous opening of chromatin in the promoter region may allow Dorsal to bind the *kBp* motif. Our results that Dorsal auto-activation is independent of co-regulator and auto-repression is dependent on its interaction with a co-repressor, AP1, support the previous findings that the Dorsal, by default, is an activator and to function as repressor it needs to interact with a co-repressor (26). To account for overall Dorsal regulation presented here, a mechanism explaining Dorsal acting as an auto-activator versus auto-repressor is warranted. On the basis of the data presented in this study, we propose that the distinction between auto-activator and auto-repressor Dorsal lies in its ability to interact with co-regulators, which also probably involves chromatin changes.

Any gene regulation mechanism that employs general factors for regulation must involve specific transcriptional regulators for spatio-temporal specificity. In yeast, for example, tissue-specific repression of *a2* promoter is regulated by its own gene in the presence of a general factor SIN4 (36). Being a chromatin modifier, SIN4 acts as a general factor, but spatial specificity is imparted by the tissue-specific transcription factor *a2*. Furthermore, GATA factors have been shown to impart tissue specificity in the expression of AMP genes (30, 31). Our result that AP1 not only acts as co-repressor of Dorsal but also imparts temporal specificity in binding of Dorsal to the *kBp* is consistent with these findings that gene expression is regulated by a general factor in combination with a specific factor. Hence, we propose that Rel proteins act as general transcription factors during immune response, and spatio-temporal specificity of Rel-mediated gene expression is imparted by other regulators like AP1 and GATA.

AP1-binding region in the *dl* promoter is A-T rich. Similar A-T rich sequence is also present upstream of the Dorsal-bind-

The requirements of *kBp*-*kB* orientation and dynamic relocalization of Dorsal from one motif to another imply a possible role of chromatin dynamics in the regulation of *dl*.

**DISCUSSION**

Conventional knowledge of autoregulation suggests that the gene product causes either auto-activation or auto-repression of its own gene. Here, while studying *dl* regulation, we have deciphered a novel mechanism of autoregulation where the gene product controls activation as well as repression of its own gene. In the study reported here, the following points have emerged. (i) *dl* autoregulation is mediated by two distinct *kB* motifs in the *dl* gene as follows: an enhancer motif present in the first intron, and a repressor motif in the promoter. (ii) The two motifs act independently to control overall regulation (initial activation and late repression) of *dl* during the course of acute phase response. (iii) *dl* activation appears to be independent of co-factor requirement; however, *dl* repression requires a co-repressor, identified here as AP1. (iv) Both AP1 and Dorsal proteins are required for the repression of *dl* at the terminal phase of acute phase response; in absence of AP1 or its binding motif, Dorsal bound to the *kBp* motif did not function as either activator or repressor. (v) Dorsal repositioning at these two motifs is temporally regulated and probably involves chromatin alteration.

FIGURE 7. **Model of Dorsal autoregulation.** Infection by Gram-positive bacteria or fungi activates the Toll-Dorsal circuit that leads to nuclear localization of transcription factor Dorsal. A, once inside the nucleus, Dorsal binds to the enhancer *kB* motif of the *dl* gene leading to induction of Dorsal synthesis thus establishing a positive feedback loop that constitutes subcircuit I (thick arrow). B, termination of immune response is marked by repositioning of Dorsal at repressor *kB* motif (dashed arrow). Binding of Dorsal to the *kB* motif is facilitated by its interaction with co-regulator AP1. Assembly of Dorsal-AP1 complex at *kB* shuts *dl* expression thus marking the termination of acute phase response. This constitutes subcircuit II of the autoregulatory loop. It is to be noted that *dl* activation is independent of any co-regulator. In contrast, *dl* repression is co-regulator-dependent (AP1 shown as diamond). Thus, both activation and repression of *dl* are autoregulated by Dorsal in a modular fashion and are temporally regulated.

The requirements of *kBp*-*kB* orientation and dynamic relocalization of Dorsal from one motif to another imply a possible role of chromatin dynamics in the regulation of *dl*. The requirements of *kBp*-*kB* orientation and dynamic relocalization of Dorsal from one motif to another imply a possible role of chromatin dynamics in the regulation of *dl*.
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...ing site in the zen promoter (37). Deletion or point mutation in the A-rich sequence of the zen promoter turns Dorsal into an activator. Although the nature of the putative co-repressor has remained uncharacterized, its physical interaction with Dorsal was established (37). Together with the findings of Kirov et al. (37), the results presented here highlight the role of cis-motifs proximal to Dorsal-binding sites in co-regulation of Dorsal.

Our results demonstrate that the κB motif is a general enhancer motif. However, dl autorepression requires not only binding of Dorsal to the κB motif but also its interaction with AP1 (Fig. 6). The motif-swapping experiments demonstrate that AP1 interaction was specific for the κB motif (Figs. 4–6). It raises the following question: how does only the κB motif but not in the other. Taken together, these results prompt us to raise the question: how does only the κB motif but not in the other. Taken together, these results prompt us to...