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

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Nirotpal Mrinal[1] **and Javaregowda Nagaraju**[2]

From the Laboratory of Molecular Genetics, Centre for DNA Fingerprinting and Diagnostics, Tuljaguda, Nampally, Hyderabad, Andhra Pradesh 500001, India

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, κB^I (intronic κB) and κB^P (promoter κB), present in the *dorsal* gene. Although the κB^I motif functions as an enhancer, the κB^P motif acts as a transcriptional repressor. Interestingly, Dorsal binding to these two motifs is dynamic; immediately upon immune challenge, Dorsal binds to the κB^I leading to auto-activation, whereas at the terminal phase of the immune response, it is removed from the κB^I and repositioned at the κB^P, resulting in its repression. Furthermore, we show that repression of Dorsal as well as its binding to the κB^P 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) Dl-κB^I for activation and (ii) Dl-AP1-κB^P 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 (Dl) 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

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[1] Recipient of Research Fellowship from Council for Scientific and Industrial Research, Government of India.

[2] Supported by a Centre of Excellence grant from Department of Biotechnology, Government of India. To whom correspondence should be addressed. E-mail: jnagaraju@cdfd.org.in.

[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.

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 *dorsal* (*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 κ*B* sites, a canonical κ*B* motif (κB^I) located in the first intron of *dl* and another functional but noncanonical κ*B* motif (κB^P) present upstream of the transcription start site (TSS). We show that the κB^I motif acts as an enhancer, whereas the κB^P 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 κB^I motif, which results in immediate and strong expression of *dl* gene. However, later in acute phase response, Dorsal binding was located at the κB^P motif and not at the κB^I 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 Jra (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^1 flies were obtained from Bloomington Stock Centre. All stocks were maintained and the experiments performed at 25 °C. *Drs*::*gfp* transgenic flies were provided by Bruno Lemaitre, 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 $MgCl_2$, 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-^{32}P]ATP (5×10^5 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 ^{32}P-labeled double-stranded synthetic oligonucleotide probe (4000 cpm/μl), 10 μl of nuclear extracts, and 300 ng of poly(dI-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 κB^P, ATGAGTCACAGAAAAACAAGAAAAACA; for mut-κ*B*, ATGAGTCACAGAATAATCCAGAATAATCC, and for κB^I, GGGAATTCCGGGAATTCCGGGAATTCC.

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, Mg^{2+}, 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 $Mg(CH_3COO)_2$, 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-

ceau S (Sigma) and blocked in 10% nonfat dry milk in 0.5% Tween 20, 0.05% SDS in PBS (Blocking buffer). The blots were incubated for 6 h at room temperature in primary antibodies and then washed four times for 10 min in Tween 20 + PBS followed by a 2-h incubation at room temperature with the secondary antibody (Sigma). The blots were then washed three times for 30 min in Tween 20 + PBS and rinsed once in PBS. Anti-Dorsal monoclonal antibody in a 1:500 dilution was used for probing. The protein bands were detected using horseradish peroxidase-enhanced chemiluminescence (ECL, Amersham Biosciences).

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 pGL3 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 $\Delta\kappa B^P$ plasmid, the NsiI recognition sequence surrounding the κB^P 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 κB^P motif harboring NsiI recognition sequence on either side of the mutant kappa*B* motif sequence (AGAATAATC) in *P3-Ex1-In1-Ex2* plasmid that generated the plasmid $\kappa B^{P\text{mut}}$. For motif swap experiment, the κB^I motif was cloned into the NsiI sites surrounding the κB^P motif. To generate $\kappa B^{I\text{mut}}$ plasmid, the mutant κB^I motif, GGGAAATAC, was used to replace the wild type κB^I (GGGAATTCC) by site-directed mutagenesis using QuickChange II site-directed mutagenesis kit (Stratagene) according to manufacturer's protocol. The mutated nucleotides of the κB^I 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 MmeI only or both MmeI and NaeI. All the plasmids were purified using Qiagen columns.

Luciferase Assay—*Drosophila* immune-competent Schneider (S2) cells were maintained at 25 °C in Schneider's Insect Cell Media (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen). For transfections, cells were seeded in 6-well plates at a density of 1×10^6 cells/ml. A day later, transfection was carried out using FuGENE transfection reagent (Roche Applied Science) without removing the plasmids. 12 h post-transfection, 50 μg of LPS (Sigma) and 50 μg of PGN (Sigma) were added per well, and the cells were harvested at different time points. Cell extracts were prepared in lysis buffer (Promega), and luciferase activity was measured according to manufacturer's instructions (Promega) on a luminometer. For luciferase assay, 100 μl of the reaction mixture/cell extract was added to 500 μl of luciferase reagent at room temperature. Luminometer was programmed to perform a 2-s measurement delay followed by a 10-s measurement.

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 T_7 and SP_6 RNA polymerases and annealed after ethanol precipitation. For RNAi, 1×10^6 *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.

Chromatin Immunoprecipitation (ChIP)—The protocol followed for ChIP was essentially as mentioned on the flychip website with the following modifications. S2 cells were fixed by cross-linking with 1% formaldehyde followed by lysis in SDS lysis buffer and sonication. Fragmented chromatin was centrifuged at 13,000 rpm for 45 min; soluble fraction was collected and later used for immunoprecipitation performed with anti-Dorsal monoclonal antibody. 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 κB^I motif, forward primer CAAAGAAAATGGAGGGCAGA and the reverse primer AAGAGAGAGTGGGCAAAGAGC. This primer pair amplifies 177-bp PCR product.

Primers for amplifying κB^P motif forward primer TTGGTTACCATACAGTTGAATTCTCA and reverse primer AGGAATGCAGGCCAGTTGTT amplify a 196-bp PCR product. Both primer sets were standardized to amplify at T_m 62 °C and were thus used in multiplex PCR.

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

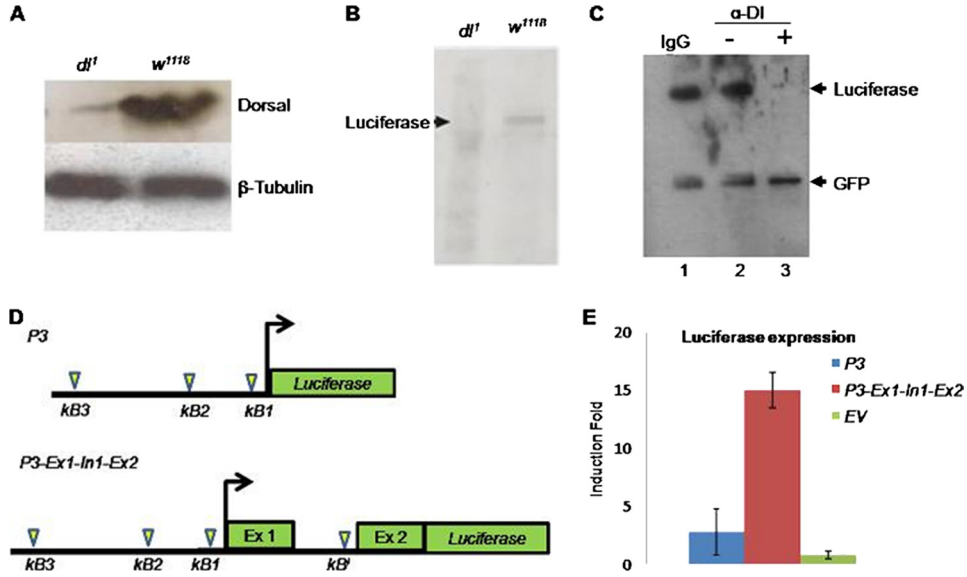

FIGURE 1. ***dl* gene is autoregulated.** *A*, absence of Dorsal in dl^1 mutant flies compared with w^{1118}, as seen in the Western blot, indicates deregulation of *dl* gene. Embryonic extracts from w^{1118} and dl^1 mutant were transferred to a nylon membrane and probed with anti-Dorsal monoclonal antibody. *Drosophila* β-tubulin was used as loading control. *B*, to investigate regulation of *dl* promoter, a full-length regulatory region of *dl* was cloned upstream of luciferase reporter and subjected to coupled in vitro transcription and translation reaction. Western blot of the reaction product shows lack of luciferase synthesis in dl^1 embryonic extract but not in w^{1118} extract when probed with anti-luciferase antibody. *C*, to confirm *dl* autoregulation, Dorsal protein from the wild type *Drosophila* was immunodepleted, and the Dorsal-depleted extract was used for coupled *in vitro* transcription and translation of the full-length reporter plasmid *P3-Ex1-In1-Ex2::luciferase* as shown in *B*. Immunodepletion of Dorsal (*lane 3*) from w^{1118} embryonic extract results in lack of luciferase synthesis. Luciferase synthesis was not affected with the embryonic extracts where either IgG was used (*lane 1*) or no antibody was added (*lane 2*). GFP synthesis was unaffected in all the three experiments. Control *actin::GFP* plasmid was simultaneously added along with *P3-Ex1-In1-Ex2::luciferase* plasmid in all three reactions. *D*, schematic showing location of putative κ*B* motifs in different *dl* constructs. Plasmid P3 harbors three upstream κ*B* motifs (κ*B1*, κ*B2*, and κ*B3*), although the plasmid *P3-Ex1-In1-Ex2* harbors additional κ*B* motif (κB^I) present in the first intron of the *dl* gene. *E*, luciferase induction upon PGN treatment was ~15-fold with *P3-Ex1-In1-Ex2* plasmid but only ~3-fold with P3 plasmid suggesting that κB^I motif has strong enhancer activity compared with the upstream three κ*B* motifs.

vicinity or far away from the κ*B* 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 κ*B* motif in their promoters; however, the same is not true for the *dl* gene, which has multiple κ*B* 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^1 mutant flies indicating absence of functional Dorsal (10, 15, 18). dl^1 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^1 flies. Interestingly, in comparison with w^{1118} flies, the level of Dorsal in extracts prepared from dl^1 embryos was extremely low or absent (Fig. 1*A*). Absence of the Dorsal protein in dl^1 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 w^{1118} embryonic extract but not with extracts from the mutant dl^1 embryos (Fig. 1*B*). This result is consistent with the result shown in Fig. 1*A* where a negligible amount of Dorsal was present in the dl^1 extract (Fig. 1, *A* and *B*) suggesting that *dl* promoter is not inducible in dl^1 embryonic extract. This result also implies that absence of Dorsal in Fig. 1*A* and luciferase in Fig. 1*B*, 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^1 embryo is *dl* deregulation and not mRNA instability. Lack of Dorsal in the dl^1 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 w^{1118} 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 w^{1118} embryonic extracts using anti-Dorsal monoclonal antibody. The Dorsal-depleted w^{1118} embryonic extract failed to synthesize luciferase upon coupled *in vitro* transcription and translation from the *P3-Ex1-In1-Ex2::luciferase* reporter plasmid (Fig. 1*C*). 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. 1*C*, *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 κ*B* motif. All the Dorsal target genes have this motif in their regulatory regions. If Dorsal regulates its own expression, then

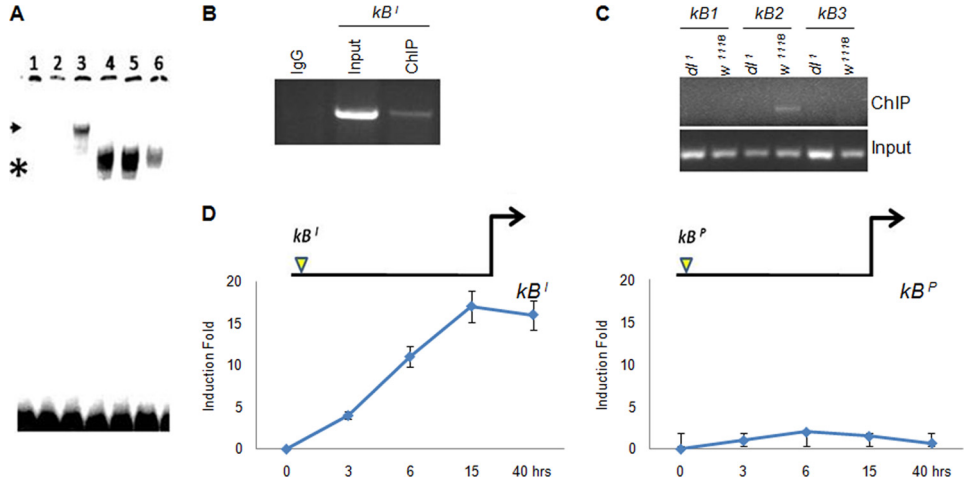

FIGURE 2. **Identification and characterization of functional κB motifs in the *dl* gene.** *A,* dorsal-specific complex is retarded upon EMSA with $κB^I$ motif as probe (marked by *asterisk, lanes 4* and *5*), which was supershifted with the Dorsal antibody (marked by *arrowhead, lane 3*). *Lane 1*, 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 w^{1118} embryo; *lane 6*, nonspecific competition. *B,* ChIP performed with anti-Dorsal antibody resulted in enrichment of $κB^I$ motif. Chromatin for ChIP was precipitated from S2 cells 15 h post-LPS + PGN 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^1 mutant. *D,* luciferase assay suggested that the $κB^I$ was inducible upon immune challenge but not the $κB^P$. The *schematics* above the *graphs* show position and identity of κB motifs used in the *dl* promoter construct.

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-In1-Ex2-luciferase* plasmid, which also includes the intronic κB motif in addition to the three upstream κB motifs (Fig. 1*D*). Strong induction of luciferase was observed with the *P3-Ex1-In1-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 autoactivation of *dl*. Strong luciferase induction from *P3-Ex1-In1-Ex2* plasmid suggested that the functional Dorsal-binding motif was present downstream of TSS in the first intron (Fig. 1*E*). Next, the ability of Dorsal to interact physically with the intronic κB motif (GGGAATTCC), named $κB^I$, was also checked by gel shift assay (Fig. 2*A*). Furthermore, supershift with anti-Dorsal monoclonal antibody confirmed that the DNA-protein complex retarded in other lanes indeed included Dorsal (Fig. 2*A*, *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 ($κB^I$) motif upon ChIP confirmed *in vivo* interaction of Dorsal with the regulatory motif $κB^I$ (Fig. 2*B*). This is consistent with luciferase reporter assay and EMSA results (Figs. 1*E* and 2*A*). 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. 2*C*). Because this was the only promoter κB motif that was precipitated upon ChIP, we named it $κB^P$. These results suggested the presence of two functional Dorsal-binding κB motifs in the *dl* gene, one in the promoter, the $κB^P$, and another one in the 1st intron, the $κB^I$ (Fig. 2*C*). Lack of enrichment of the other two promoter κB motifs suggested that they were probably not functional (Fig. 2*C*).

Because ChIP experiments identified two Dorsal-binding motifs in the *dl* gene, we next tested how these two motifs regulated *dl* expression. Although the $κB^I$ motif GGGAATTCC is a typical κB motif, the $κB^P$ 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 $κB^P$ and $κB^I$ individually in the same *P3* promoter construct. For comparison, the $κB^P$ motif in the *P3* plasmid was replaced with the $κB^I$ motif so that the two plasmids differed only in their Dorsal-binding sequences. Strong luciferase induction was observed with the *P3-*$κB^I$ plasmid but not with the *P3-*$κB^P$ plasmid under the same experimental conditions (Fig. 2*D*). In fact, luciferase induction with the *P3-*$κB^I$ promoter was comparable with that of the full-length construct *P3-Ex1-In1-Ex2,* suggesting the contribution of the $κB^I$ motif in Dorsal activation (Figs. 1*E* and 2*D*). If the $κB^P$ motif had no role in activation of *dl,* then what was the significance of its interaction with the Dorsal?

Dorsal Binding to $κB^P$ *and* $κB^I$ *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 $κB^I$ motif, which explains Dorsal activation upon immune challenge (Fig. 3*A*). However, around 36 hpi, when the immune response had reached terminal stage, Dorsal binding was seen at the $κB^P$ motif but not at the $κB^I$ motif (Fig. 3*A*). 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-

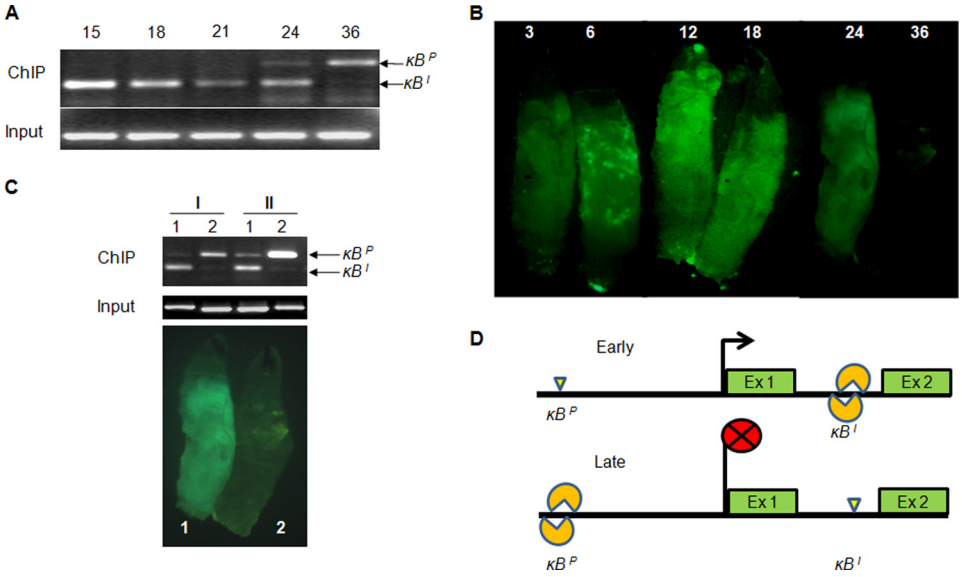

FIGURE 3. **Dorsal is repositioned to the κB^P motif from the κB^I motif during the course of immune response.** *A,* ChIP suggests that Dorsal binds to the κB^I motif at the onset of immune response and remains bound until ~30 hpi in immune activated S2 cells. During this period, the κB^P motif does not appear to be occupied by Dorsal. However, Dorsal binding to the κB^P and not to the κB^I motif is seen around 36 hpi. At 24 hpi, bands corresponding to κB^P (196 bp) as well as κB^I (177 bp) motifs are amplified indicating a transition stage when Dorsal is bound to both the motifs. Single band in the *input panel* corresponds to the κB^I locus. *B,* time course of *drosomycin* expression in *Drs::GFP* larvae upon bacterial infection shows AMP expression profile during a typical acute phase response. *C,* repositioning of Dorsal from its binding site in the intron to the other binding site in the *dl* promoter was seen in *Drosophila* larvae too, as evidenced by ChIP assay. *I* and *II* represent two independent ChIP experiments performed in bacteria-challenged *Drosophila* larvae. *Lane 1,* chromatin precipitated 18 hpi when GFP expression was high; *lane 2,* chromatin isolated 48 hpi when GFP expression had died down signifying termination of immune response. *D,* schematic shows repositioning of Dorsal from the κB^I to the κB^P motif during immune response. Binding of Dorsal to the κB^I is an early event (*Early*) that leads to induction of the *dl*. At a later stage of immune response (*Late*), Dorsal is removed from the κB^I motif and repositioned at the κB^P.

ulation by the two autoregulatory *κB* motifs is their temporal delineation in binding to Dorsal during acute phase response.

We also validated these results *in vivo* by performing ChIP on nuclear extracts isolated from fat body of bacteria-challenged *Drosophila* larvae at 15 and 48 hpi (Fig. 3, *B* and *C*). In challenged larvae, Dorsal occupied the κB^I motif at 15 hpi, and by 48 hpi, when the immune response had almost died down, Dorsal was found to be associated with the κB^P motif (Fig. 3, *B* and *C*). These results also suggest that binding of Dorsal to the two autoregulatory *kB* motifs in the *dl* gene is a dynamic process as Dorsal initially binds to the κB^I motif and later gets repositioned at the κB^P motif (Fig. 3*D*).

Distinct Orientation of κB^P and κB^I Motifs Is Crucial for Dorsal Regulation—Although the κB^P motif does not appear to control *dl* induction *in vitro* (Fig. 2*D*), its interaction with Dorsal *in vivo* only in the dying stages of acute phase response indicated its probable role in *dl* regulation. However, luciferase reporter experiment with individual *κB* motifs did not reveal any conclusive role of κB^P in transcriptional regulation of *dl* (Fig. 2*D*). Hence, we set out to investigate the functional significance of Dorsal binding to the κB^P motif (Fig. 2, *C* and *D*). Because Dorsal binding the two motifs is temporally exclusive, we decided to study its significance by sequentially replacing and swapping the two motifs in the full-length construct *P3-Ex1-In1-Ex2*.

P3-Ex1-In1-Ex2 plasmid was transfected into S2 cells, followed by immune induction by LPS + PGN treatment. A time 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. 4*A*). For the next experiment, we generated a κB^P-κB^P construct, by replacing the κB^I motif with the κB^P, and found that this construct hardly showed any luciferase induction (Fig. 4*B*). In another experiment, the κB^P motif in the control plasmid was replaced with the κB^I; the resultant luciferase-reporter construct, harboring two κB^I motifs (κB^I-κB^I), caused strong luciferase induction and remained constitutively active (Fig. 4*C*). These results demonstrated the enhancer property of κB^I motif in the regulation of *dl*. Next, we swapped the two *κB* motifs; the resultant new plasmid had κB^I motif in the promoter and κB^P motif in the first intron (κB^I-κB^P plasmid). We found that κB^I-κB^P construct was constitutively active, and there was no decrease in luciferase synthesis by 40 hpi as observed with the control κB^P-κB^I construct (Fig. 4, *A* and *D*).

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

The motif-swapping experiment further revealed that it is not only the presence of the two *κB* motifs but also their arrangement in the *dl* gene that is important for *dl* autoregulation during acute phase response. In other words, the κB^P-κB^I arrangement (where κB^P is present upstream of TSS and κB^I 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 κB^I and not to the κB^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 κB^P motif, and possibly time-dependent recruitment of Dorsal to κB^P requires participation of other proteins and/or chromatin changes.

Dorsal Recruitment to κB^P Motif Requires AP1 as Co-regulator—The motif swap experiment suggested the requirement of κB^P-κB^I arrangement for control of the expression dynamics of *dl*, where κB^P motif probably brings about the time-dependent repression of *dl*, although initial induction is controlled by the

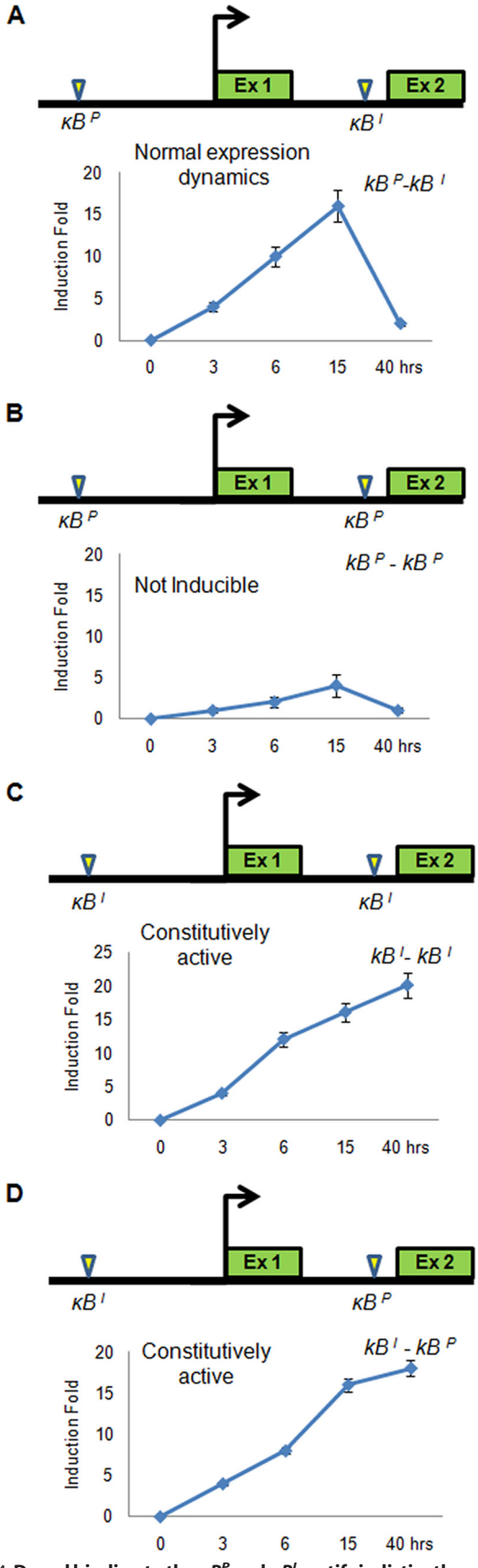

FIGURE 4. **Dorsal binding to the κB^P and κB^I motifs is distinctly regulated.** *A*, luciferase expression construct mimicking the κB^P-κB^I organization in the *dl* gene shows rapid induction of luciferase expression followed by its repression by 40 hpi. *B*, replacing the κB^I with the κB^P resulted in κB^P-κB^P organization that was hardly inducible. *C*, reporter construct where the κB^P was replaced with the κB^I and thus had two κB^I motifs (κB^I-κB^I) and remained constitutively active. *D*, when the κB^P and κB^I motifs in the reporter κB^P-κB^I plasmid were swapped, the resultant κB^I-κB^P plasmid remained constitutively active. These results suggest that κB^P-κB^I 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*.

κB^I motif. This raised the intriguing question of how Dorsal recruitment to κB^P 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 κB^P 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 κB^P or κB^I motifs, we performed EMSA using κB^P and κB^I motifs as probes, which revealed retardation of complexes of different sizes (Fig. 5*A*). The protein complex recruited at the κB^P motif was bigger in size compared with the complex retarded with the κB^I motif, suggesting that one or more additional proteins were present in the Dorsal complex retarded with the κB^P motif (Fig. 5*A*). Next, we investigated the following: (i) the identity of the co-regulatory protein(s) involved in Dorsal binding to the κB^P motif, and (ii) whether the difference in protein-protein interaction can explain the difference in spatio-temporal regulation of *dl* by κB^P and κB^I 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 κB^P 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 κB^P motif in the *dl* promoter (Fig. 5*B* 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. 5*C*). 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 *P3-Ex1-In1-Ex2* plasmid. Thus, generated AP1-del luciferase reporter plasmid was constitutively active, whereas luciferase expression from the control *P3-Ex1-In1-Ex2* plasmid underwent time-dependent repression (Fig. 5*D*). To confirm the probable cross-talk between AP1 and Dorsal, we mutated the κB^P motif in the *P3-Ex1-In1-Ex2* plasmid to generate κB^P-*mut* plasmid. Luciferase expression from the κB^P-*mut* construct was also constitutive similar to that of AP1-del construct (Fig. 5*D*). Constitutive expression of luciferase upon deletion of the AP1 cluster or mutation of the κB^P motif suggests that both κB^P and AP1-binding elements may be required for time-dependent *dl*

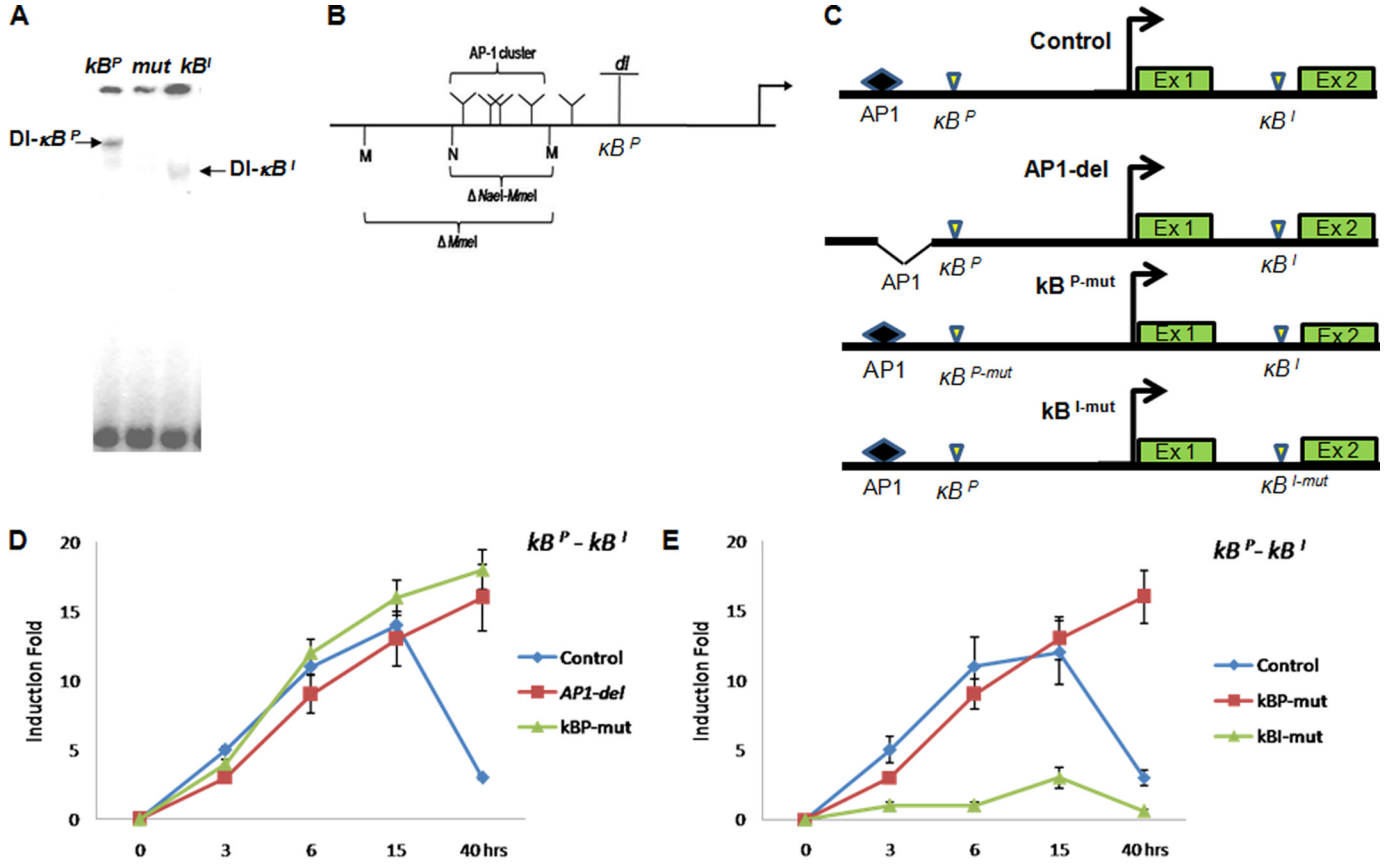

FIGURE 5. **Dorsal binding to the κB^P motif is co-regulator-dependent.** *A*, EMSA was performed with the κB^I and κB^P motifs as probes. κB^P (AGAAAAACA) retards a larger complex (*1st lane*) compared with the κB^I (GGGAATTCC) (*lane 3*). *2nd lane* (*mut*) shows competition with mutant oligonucleotide (mut-AGAATAATCC) where no Dorsal complex is retarded. *B*, diagrammatic representation of clustered AP1-binding sites in the *dl* promoter upstream of κB^P. Restriction enzymes used to delete the AP1 cluster are also shown. *C*, schematic representation of different reporter plasmids used for luciferase assay in experiments as explained in *D* and *E*. *D*, deletion of AP1 cluster in the *dl* promoter leads to constitutive expression of luciferase compared with control full-length reporter plasmid indicating the role of AP1 in *dl* repression. *E*, mutation in the κB^P motif also leads to constitutive expression of luciferase suggesting that Dorsal binding to the κB^P is also critical for *dl* repression. However, when the κB^I was mutated, no induction of luciferase was seen suggesting that the κB^I was required only for transcriptional activation of dl. The κB^P-κB^I promoter construct was used as the control plasmid.

repression during acute phase response (Fig. 5*D*). On the other hand, when the κB^I was mutated (κB^I-*mut* plasmid), no significant luciferase induction was observed at any time point, suggesting that the κB^I motif is required for the *dl* activation (Fig. 5*E*). These results are consistent with motif swap experiments where replacing the κB^P motif with the κB^I also resulted in constitutive expression of luciferase (Fig. 4*D*). Thus, our data confirm that the κB^I is an enhancer motif and is required for the initial induction of *dl* (Figs. 5*D* and 3, *A–D*). On the other hand, the κB^P 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 κB^P Motif Leads to dl Repression—To further dissect the role of AP1-Dorsal interaction in *dl* regulation, we took to the RNAi approach. The κB^P-κB^I 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. 6*A*). However, when κB^I-κB^P plasmid was used for reporter assay in the presence of *AP1-dsRNA*, there was no repression of luciferase activity (Fig. 6*B*). These results further suggest the following: (i) κB^I is an enhancer motif, and (ii) κB^P 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 κB^P motif with respect to κB^I motif (Fig. 5*A*). We speculated that the larger Dorsal-κB^P complex probably also contained AP1 proteins apart from Dorsal. To test such a possibility, we performed EMSA using κB^P as probe with whole and AP1-depleted nuclear extracts. A clear shift in gel retardation was seen between control (Fig. 6*C*, *lane 1*) and AP1-depleted nuclear extracts (Fig. 6*C*, *lanes 2–5*). Depletion of AP1 resolves the control band (Fig. 6*C*, *lane 1*) into two (*lanes 2–5*) suggesting that AP1 interacts with the Dorsal-κB^P complex (Fig. 6, *A* and *B*). Depletion of AP1 by RNAi was also verified by EMSA, which showed progressive loss of AP1-specific band (Fig. 6*D*).

ChIP results and RNAi data together suggest that AP1 action is seen after 30 hpi (Figs. 6*A*, 2*C*, and 3, *A* and *C*). This may explain the recruitment of Dorsal to the κB^P motif late in immune response but not in early stages. To follow the time-dependent assembly of the Dorsal-AP1 complex on the κB^P

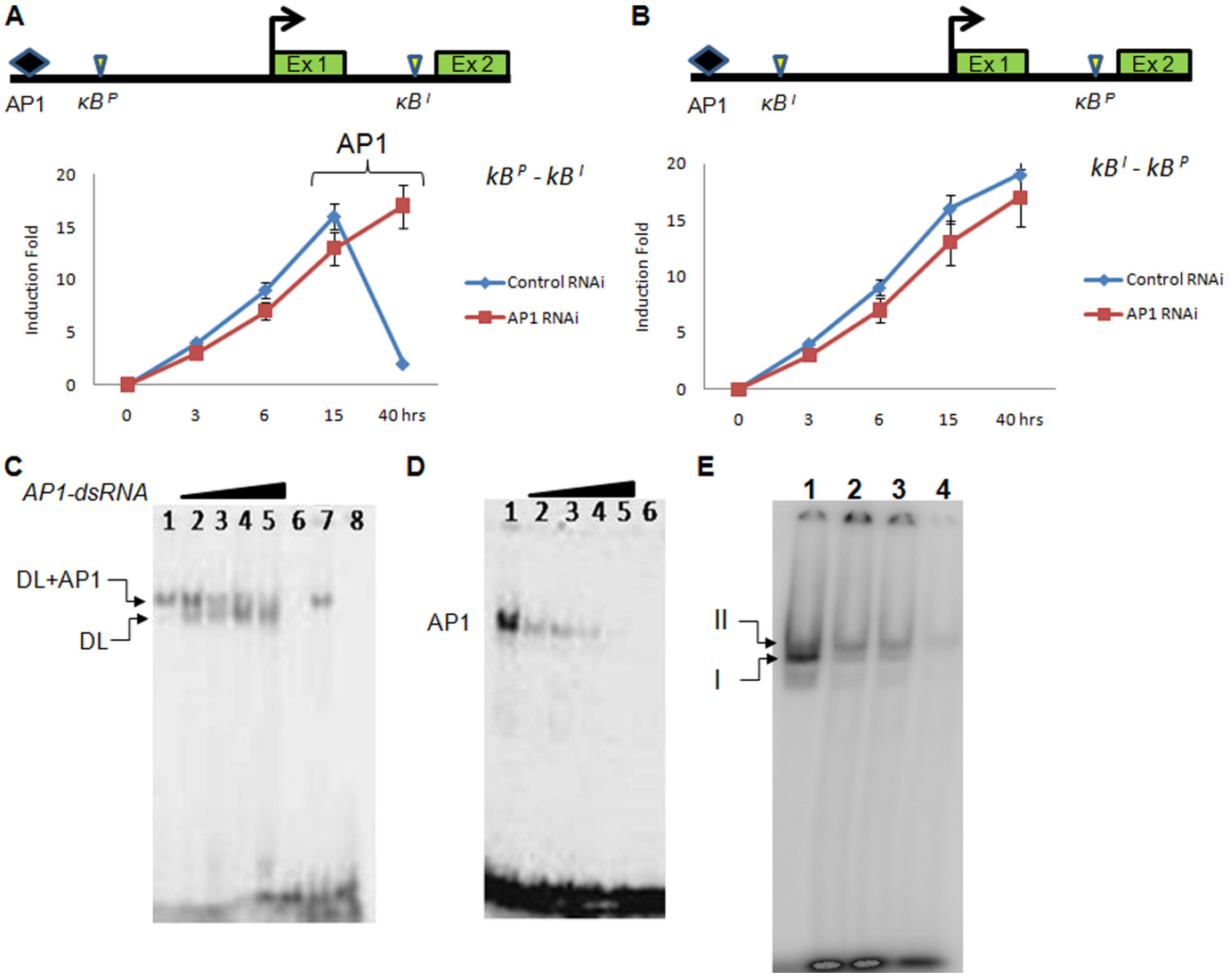

FIGURE 6. **Recruitment of AP1-Dorsal complex on the κB^P motif controls *dl* repression.** *A,* depletion of AP1 by RNAi abolishes *dl* repression with κB^P-κB^I construct but does not affect its activation. Effect of depletion of AP1 on *dl* repression is seen only in the later phase of immune response, although AP1-dsRNA is present throughout the experiment. *B,* no effect of AP1 depletion on *dl* repression was seen with κB^I-κB^P plasmid (where the two motifs have been swapped) suggesting specific requirement of the κB^P in the vicinity of the AP1-binding motif in the *dl* promoter. *C,* depletion of AP1 by RNAi resolves the larger complex of Dorsal (*DL*) and AP1 (*lane 1*) into smaller one of Dorsal only (*lanes 2–5*), suggesting assembly of both Dorsal and AP1 proteins as a complex on the κB^P motif. *Lane 1,* mock transfection; *lanes 2–5,* increasing amount of AP1-dsRNA; *lane 6,* cold competition; *lane 7,* nontarget RNAi; *lane 8,* mutant κB^P oligonucleotide. *D,* specific depletion of AP1 by RNAi was confirmed by EMSA. Nuclear extracts used in *lanes 1–6* are the same as in *C*. *E,* dorsal interaction with AP1 and their recruitment on the κB^P motif is time-dependent as seen in EMSA. *I* and *II* denote two different sized complexes (*complex I* = Dorsal alone and *complex II* = Dorsal-AP1). Nuclear extracts prepared at different time points post-PGN + LPS treatment of S2 cells were incubated with radiolabeled κB^P and κB^I probes together and resolved by EMSA. *Numbers above* each lane indicate nuclear extracts isolated at four different time points from immune-challenged S2 cells. *Lanes 1,* 15 hpi; *lane 2,* 30 hpi; *lane 3,* 36 hpi; *lane 4,* 40 hpi.

motif, we performed competitive EMSA where the κB^P and κB^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 κB^P and κB^I could be resolved in the same lane. Fig. 6*E*, *lane 1* (where nuclear extract isolated at 15 hpi was used), shows a strong retardation of a smaller complex of Dorsal bound to κB^I. However, with nuclear extracts isolated 24, 30, and 40 hpi (Fig. 6*E, lanes 2–4*), retardation of a higher size complex corresponding to Dorsal bound to κB^P is also seen. Results of competitive EMSA further emphasize that interaction of Dorsal with the two κB motifs is dynamic and temporally regulated.

Overall, our data suggest that the κB^I motif controls the induction of *dl* seen in immune-challenged *Drosophila,* whereas the κB^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 κB motifs. During the late phase of immune response, Dorsal is removed from the κB^I motif (Figs. 4, *A–D*, 5*D,* and 6*D*) and is repositioned at the κB^P motif. We have shown that the κB^P motif on its own is not a repressor motif (Figs. 2*D* and 3*B*), 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 κB motifs in a κB^P-κB^I orientation (Fig. 3, *A–D*).

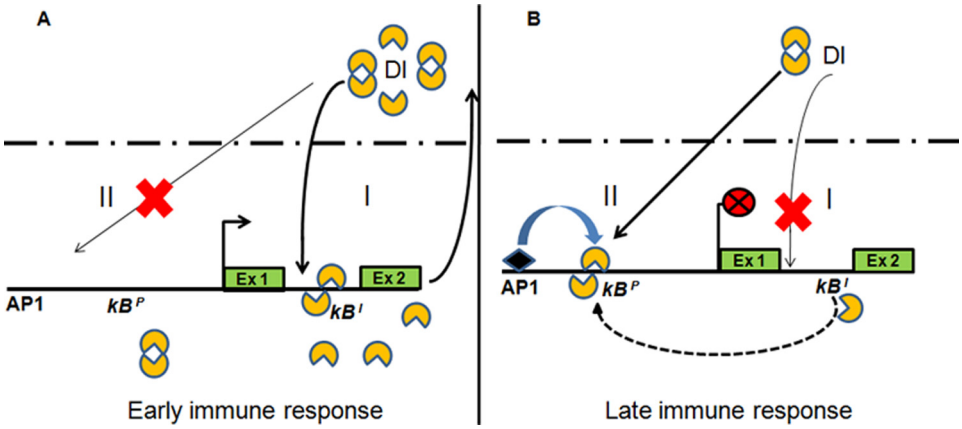

FIGURE 7. **Model of Dorsal autoregulation.** Infection by Gram-positive bacteria or fungi activates the Toll-Dorsal circuit that leads to nuclear localization of transcription factor Dorsal. *A,* once inside the nucleus, Dorsal binds to the enhancer κB^I motif of the *dl* gene leading to induction of 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 κB^P motif (*dashed arrow*). Binding of Dorsal to the κB^P motif is facilitated by its interaction with co-regulator AP1. Assembly of Dorsal-AP1 complex at κB^P 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 κB^P-κB^I orientation and dynamic relocation 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 κB 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 the absence of AP1 or its binding motif, Dorsal bound to the κB^P motif did not function as either activator or repressor. (v) Dorsal repositioning at these two motifs is temporally regulated and probably involves chromatin alteration.

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,κB^I. 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. 7*A*). 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-Dorsal-κB^P gets activated and causes removal of Dorsal from the κB^I motif and its recruitment to the κB^P motif.

Our data indicate that Dorsal bound to the κB^I 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 κB^P 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 κB^P motif in the promoter and contraction of chromatin near the κB^I motif in the first intron. As a result, the κB^I motif would become inaccessible to Dorsal. Simultaneous opening of chromatin in the promoter region may allow Dorsal to bind the κB^P 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 α2 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 α2. 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 κB^P 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-

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^I motif is a general enhancer motif. However, *dl* autorepression requires not only binding of Dorsal to the κB^P but also its interaction with AP1 (Fig. 6). The motif-swapping experiments demonstrate that AP1 interaction was specific for the κB^P motif (Figs. 4–6). It raises the following question: how does only the κB^P motif facilitate cross-talk between Dorsal and AP1 but not the κB^I motif? It is pertinent to note that the repressor motifs κB^P of *dl* (AG*AAAAA*CA) and *zen* promoter (GGAAAATCC) have an A-rich core (37). It is known that a continuous stretch of four or more "A" nucleotides induces bending in the DNA (38). Furthermore, biophysical analyses of different *κB* motifs suggest that A-tract imparts a flexible conformation that may favor Dorsal-co-regulator interaction compared with typical *κB* motifs.[4] This may explain why the AP1-κB^P motif in *dl* promoter interacts in *cis* but not when the κB^I motif is placed next to AP1 motif in the same *dl* promoter (Figs. 4–6).

Another example of tissue-specific regulation by an atypical A-rich *kB* motif comes from the regulation of *iNOS* gene by NF*κ*B protein (39). Human *iNOS* gene has multiple *κB* motifs, and the one present 6.4 kb upstream has an A-rich core (GGAAAAACC), similar to the κB^P motif. The GGAAAAACC motif of *iNOS* was functional in A549 cells but not in AKN-1 cells unlike the other four *κB* motifs that were functional in both the cell types (39). The tissue-specific activation/silencing of GGAAAAACC motif of *iNOS* may be due to interaction of NF*κ*B with a co-regulator that may be present in one cell type but not in the other. Taken together, these results prompt us to hypothesize that A-rich *κB* motifs might be more amenable to facilitating Rel interactions with co-regulators. In conclusion, our study demonstrates that Dorsal autoregulation constitutes a regulatory feedback loop through two subcircuits during immune response. Currently, we are testing this model of *dl* regulation in early embryonic development. However, it remains to be investigated whether the kinetics of activation of the two subcircuits also depends on the amount of Dorsal. Importantly, we have shown context-dependent repositioning of one regulator to two different *cis*-elements in the same gene leading to different phenotypes. Our study suggests that autoregulation can be a dynamic process that allows the regulator to interact with co-regulator(s) as well as different *cis*-elements, separated in space and time, leading to distinct phenotypes.

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[4] N. Mrinal, A. Tomar, and J. Nagaraju, submitted for publication.

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