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Immunity Regulatory DNAs Share Common Organizational Features in *Drosophila*

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Summary

Infection results in the rapid activation of immunity genes in the *Drosophila* fat body. Two classes of transcription factors have been implicated in this process: the REL-containing proteins, Dorsal, Dif, and Relish, and the GATA factor Serpent. Here we present evidence that REL-GATA synergy plays a pervasive role in the immune response. SELEX assays identified consensus binding sites that permitted the characterization of several immunity regulatory DNAs. The distribution of REL and GATA sites within these DNAs suggests that most or all fat-specific immunity genes contain a common organization of regulatory elements: closely linked REL and GATA binding sites positioned in the same orientation and located near the transcription start site. Aspects of this "regulatory code" are essential for the immune response. These results suggest that immunity regulatory DNAs contain constrained organizational features, which may be a general property of eukaryotic enhancers.

Introduction

Innate immunity is conserved in most or all metazoans. It mediates the detection and destruction of pathogenic agents within minutes or hours after infection (reviewed by Carroll and Janeway, 1999; Hultmark, 2003). Unlike adaptive immunity, innate immunity recognizes broad classes of pathogens and does not require the clonal expansion of lymphocytes bearing specific antigens. Insects such as the fruitfly, *Drosophila melanogaster*, rely solely on innate immunity for defense against infections. Two major signaling pathways comprise the fly's immune system: the Toll pathway and the PGRP-LC/*imd* pathway (Lemaitre et al., 1996; Choe et al., 2002; Corbo and Levine, 1996; Georgel et al., 2001). The Toll pathway is activated by gram-positive bacteria and fungi, while the PGRP-LC/*imd* pathway is triggered by gram-negative bacteria. The activation of these pathways leads to the transcription of antimicrobial peptide genes in the fly fat body, a lobed organ analogous to the mammalian liver. In addition to these peptides, many other gene families are coopted in the antimicrobial response, including serine proteases, iron transporters, coagulation factors, and transcription effectors. In fact, microarray studies indicate that as many as 400 genes, or roughly 3% of the entire fly genome, show changes in transcriptional activity upon infection (De Gregorio et al., 2001).

Several transcription factors have been implicated in the immune response, including Dorsal, Dif, Relish, and Serpent. Dorsal, Dif, and Relish are members of the well-characterized Rel/NF-κB family of transcription factors (reviewed in Govind, 1999). These factors contain a conserved ~300 amino acid Rel domain that functions in DNA binding, dimerization, and nuclear localization. Rel factors exist as homo- or heterodimers that are sequestered by cytoplasmic inhibitors. Dif and Dorsal are associated with the IκB homolog Cactus (Wu and Anderson, 1998; Geisler et al., 1992), while Relish contains six C-terminal ankyrin repeats that resemble those in IκB proteins (Dushay et al., 1996). Cactus is degraded and the C terminus of Relish is cleaved upon induction of the immune response. As a result, Dif and Relish enter nuclei and activate immunity genes. Dorsal is active in the early embryo where it establishes several tissues across the dorsal-ventral axis (Anderson and Nusslein-Volhard, 1984). Dorsal is also expressed in the fat body and might contribute to the immune response, although it is dispensable (Meng et al., 1999). Conversely, Dif and Relish are dispensable for development but essential for immunity (Ip et al., 1993; Hedengren et al., 1999).

Serpent is a member of the GATA family of zinc finger transcription factors, all of which recognize variations in the following sequence motif: (A/T) GATA (A/G) (reviewed by Patient and McGhee, 2002). Serpent is essential for the differentiation of the fat body and blood cells (Sam et al., 1996; Rehorn et al., 1996) and is also required for the activation of gene expression in the mature fat body during larval development. For example, Serpent is an activator of the fat body protein 1 (fbp1) gene (Brodu et al., 2001) and Cecropin A1 (CecA1), which encodes an antimicrobial peptide (Petersen et al., 1999). Altogether, six immunity regulatory DNAs have been identified: Attacins A and B (Dushay et al., 2000), Cecropin A1 (Engstrom et al., 1993), Diptericin (Reichhart et al., 1992), Drosomycin (Ferrandon et al., 1998), and Metchnikowin (Levashina et al., 1998). There is evidence that REL sites are necessary for Diptericin expression (Kappler et al., 1993), while REL and GATA sites are both required for the induction of CecA1 (Kadalayil et al., 1997).

In the present study we provide evidence that REL-GATA synergy is not restricted to the regulation of CecA1 expression but is a pervasive feature of immunity genes. Moreover, the REL and GATA binding sites exhibit a fixed organization in a variety of immunity regulatory DNAs. SELEX assays were used to identify Dorsal, Dif, Relish, and Serpent binding sites. The 5′ regulatory regions of the 50 most strongly activated immunity genes were examined for the presence of these sites. Nearly half contain three shared features. First, REL and GATA binding sites are located near the transcription start site, usually within 200 or 300 bp. Second, the REL and GATA binding sites are closely linked to one another, most often within 50 bp. Third, the REL and GATA sites are in the same relative orientation. Minimal

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regulatory DNAs were defined for Attacin A, CecA1, Diptericin, Drosomycin, and Metchnikowin. Additional regulatory DNAs were identified for PGRP-SB1, Defensin, Ady, and Runt. The detailed analysis of the Metchnikowin regulatory DNA demonstrates the importance of both REL and GATA sites for gene activation. A modified CecA1 regulatory DNA with an inverted GATA site no longer mediates activation in the larval fat body upon infection. The Defensin regulatory DNA mediates induction in the gut, but a single nucleotide substitution that creates an appropriately positioned GATA site causes the modified regulatory DNA to be active in the fat body. We discuss the basis for REL-GATA synergy and suggest that immunity regulatory DNAs contain an organization that is somewhere between the loose clustering of *cis*-regulatory elements seen for enhancers that direct stripes and bands of gene expression in the early embryo, and the rigidly organized enhanceosomes seen for the mammalian IL-2 and interferon-β genes (reviewed by Merika and Thanos, 2001).

Results

SELEX Assays

The identification of new immunity regulatory DNAs was facilitated by the characterization of Dif, Dorsal, Relish, and Serpent binding sites. For this purpose we employed SELEX (systematic *e*volution of *l*igands by *ex*ponential enrichment) assays (reviewed in Gold et al., 1995). His-tagged Dorsal, Dif, Relish, and Serpent proteins were produced in bacteria and used for the binding assays. Various combinations of proteins were used, including Dorsal homodimers, Relish homodimers (aa 3–595 and aa 113–595), and Dif/Relish heterodimers. Dif homodimers were analyzed as GST fusions, since protein-DNA complexes containing His-tagged Dif were not detected (data not shown). Two truncated forms of Relish were used to represent the predominant isoforms found in infected cells. The first form contains aa residues 3–595, which resembles the protein after the C-terminal ankyrin repeats are removed. The second form contains aa residues 113–595, and represents a different putative open reading frame that also lacks the ankyrin repeats (Dushay et al., 1996).

Protein-DNA complexes obtained in the fourth round of selection are presented in Figure 1A. A mixture of Dif and Relish produces protein-DNA complexes that are intermediate in size between Dif-DNA and Relish-DNA complexes (compare lane 4 with lanes 2 and 3, and lane 6 with lanes 2 and 5). DNA was extracted from the protein-DNA complexes, cloned and sequenced. Consensus binding sites were compiled from at least 30 sequences per protein and are presented in Figures 1B–1E.

Dorsal recognizes a sequence that is similar to the canonical NF-κB binding site, characterized by an A/T-rich core flanked by at least 3 guanines at the 5′ end and one or more cytosines at the 3′ end (Lenardo and Baltimore, 1989). Relish homodimers, in contrast, bind a similar but more permissive site. GST-Dif selects a highly degenerate site in which only the three invariant guanines are recognizable (data not shown). In contrast, heterodimers of Dif and Relish (either form) prefer a sequence different from the one they bind as homodimers: GGGA $^{A}/_{T}$ TC $^{C}/_{A}$ C. This sequence represents a nearly perfect palindrome with an inflection point in the center. Taken together these results imply that Dif and Relish homodimers may be flexible and thereby bind diverse DNA sequences, whereas Dif/Relish heterodimers have a more rigid structure and hence bind fewer sites. The consensus sequence for Serpent is related to the general motif seen for this family of transcription factors (WGATAR), although SELEX assays identified a more specific sequence that contains additional 3′ residues (Figure 1E). As discussed below, the characterization of this extended Serpent recognition sequence facilitated the analysis of immunity regulatory DNAs.

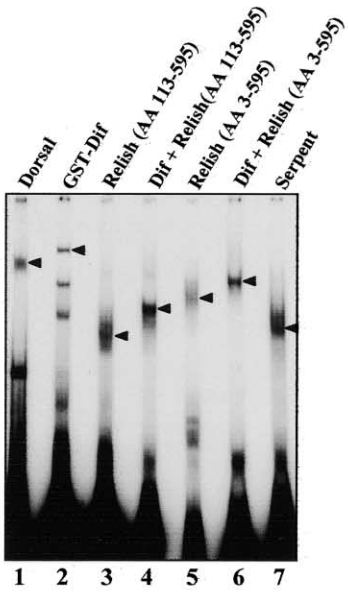
Organization of Binding Sites within Fat Body-Specific Regulatory DNAs

Microarray experiments identified 400 genes that show altered levels of expression after adult flies are infected with a cocktail of gram-negative and gram-positive bacteria (De Gregorio et al., 2001). To identify regulatory DNAs for these genes, we analyzed the 5′ flanking regions of the 50 most highly induced genes (those showing 6-fold to over 100-fold induction). These genes encode proteins that are known or predicted to function in innate immunity. We searched within 1 kb of the predicted transcription start sites for perfect matches to the Dorsal, Relish, Dif/Relish, and Serpent recognition sequences that were determined by the SELEX assays. Searches were performed using a modified version of the FlyEnhancer program that allows one to input gene names and control search window size (Markstein et al., 2002). Many of the 5′ flanking regions, 37 out of 50, contain at least one REL site and one Serpent site. Nearly half of the genes, 21 of 50, have at least one REL and one Serpent site located within 50 bp of each other and positioned in the same relative orientation. This arrangement was reported in the CecA1 regulatory DNA and is conserved in other insect species (Kadalayil et al., 1997).

The 5′ flanking regions of eight immunity genes were isolated via PCR and attached to a lacZ reporter gene (summarized in Figure 2). The binding sites for REL (shaded) and GATA (unshaded) are indicated. Palindromic REL sites are represented by doubleheaded arrows. The precise sequences and positions of the motifs relative to the predicted transcription start sites are listed in Table 1. Transgenic flies were obtained by injecting the lacZ fusion genes in P element transformation vectors. Climbing third instar larvae were poked with a needle dipped in a mixture of gram-negative and gram-positive bacteria, dissected 3–6 hr later, fixed, and then stained in a solution containing X-gal. Parallel stainings were done with uninfected larvae or larvae poked with a clean needle.

Six of the eight fusion genes mediated activation in the fat body upon infection, while two did not (Figure 3). The Lipase/lacZ fusion gene is constitutively expressed in the Malpighian tubules, while the Defensin/lacZ fusion gene mediates activation in the gut after infection. Weak staining in the fat body can be seen for the Defensin enhancer, but only after prolonged (overnight) infection. Interestingly, each of the six fat-specific

A



B **DORSAL**

POSITION:		1	2	3	4	5	6	7	8	9	
%G	60	97	100	100	0	0	0	0	0	7	30
%A	13	0	0	0	83	70	27	20	3	0	17
%T	23	0	0	0	17	27	73	53	3	37	6
%C	4	3	0	0	0	3	0	27	94	56	47
CONSENSUS:		G	G	G	A/T	A/T	T/A	no G	C	no A	

C **RELISH (AA 3-595)**

POSITION:		1	2	3	4	5	6	7	8	9	
%G	71	100	100	100	0	3	16	0	0	0	23
%A	6	0	0	0	100	55	6	3	48	0	19
%T	10	0	0	0	0	32	58	16	0	6	19
%C	13	0	0	0	0	10	20	81	52	94	39
CONSENSUS:		G	G	G	A	no G	N	C/T	C/A	C/T	

D **DIF/RELISH**

POSITION:		1	2	3	4	5	6	7	8	9	
%G	88	100	100	100	0	0	0	0	0	0	9
%A	9	0	0	0	100	56	0	0	16	3	16
%T	3	0	0	0	0	44	97	0	0	3	25
%C	0	0	0	0	0	0	3	100	84	94	50
CONSENSUS:		G	G	G	A	A/T	T	C	C/A	C	

E **SERPENT**

POSITION:		1	2	3	4	5	6	7	8		
%G	50	0	100	0	0	0	3	56	41	19	
%A	9	19	0	100	0	100	97	0	47	19	
%T	28	37	0	0	100	0	0	9	3	28	
%C	13	44	0	0	0	0	0	35	9	34	
CONSENSUS:		no G	G	A	T	A	A	no A	no T		

Figure 1. In Vitro Oligonucleotide Selection (SELEX) Assays

(A) Full-length His-tagged proteins and GST-tagged Dif were incubated with a mixture of radiolabeled oligonucleotides from fourth round SELEX oligos, and the resulting nucleoprotein complexes were separated on a native polyacrylamide gel. Arrowheads indicate complexes chosen for analysis.

(B–E) At least 30 DNAs selected from this round were cloned and sequenced. The sequences were aligned by eye, and the percent base composition for each position is given. Bases occurring in ≤3% of the sequence pool are not included in the consensus.

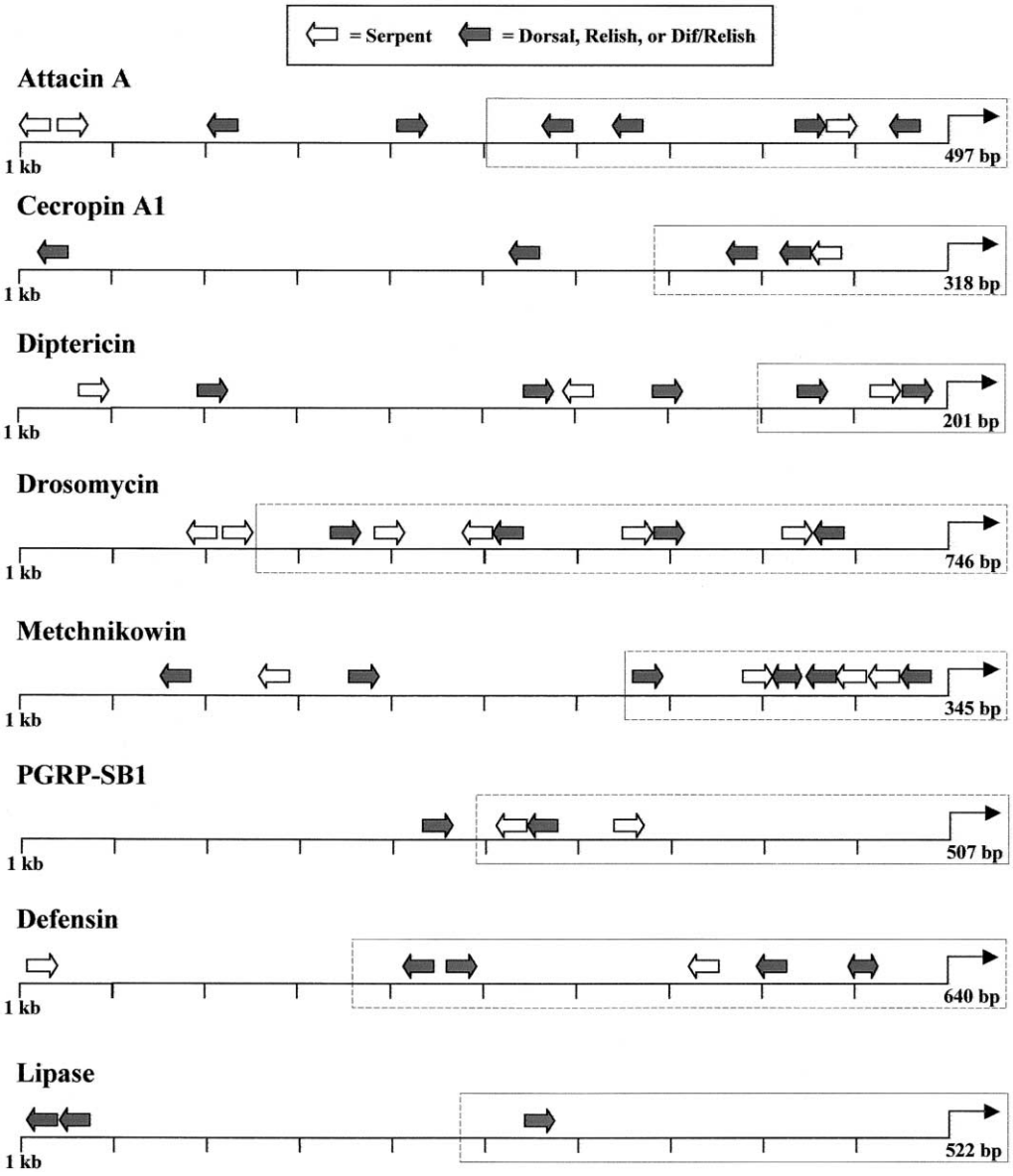


Figure 2. Distribution of SELEX Sites 1 kb Upstream of Eight Genes Induced by Infection

Perfect matches to Dorsal, Relish, and Dif/Relish matrices are designated by gray arrows, while white arrows represent matches to the Serpent consensus. Sites containing bases present at low frequency in the SELEX data (3%) are also displayed. Doubleheaded arrows represent palindromic sites. Arrows are not drawn exactly to scale. The boxed regions represent the segments tested for enhancer activity, which were chosen based on having a high density of binding sites. Tick marks represent 100 bp. The transcription start sites are those designated in GenBank.

regulatory DNAs contains closely linked REL and GATA sites (the spacing between the closest pairs range from 4 bp in Attacin A to 11 bp in PGRP-SB1). In contrast, REL and GATA sites are not closely linked in the Defensin regulatory region (they are separated by 57 bp); the Lipase regulatory region lacks a Serpent binding site. Thus, the preceding studies establish a correlation between linked REL and GATA sites and induction in the fat body.

To investigate the importance of linked REL-GATA binding sites in the immune response, we examined the activities of 16 different lacZ fusion genes that contain high-density clusters of Dorsal binding sites (Markstein et al., 2002). While Dorsal is not essential for the immune response, its consensus sequence overlaps the spectrum of sites recognized by Relish and Dif/Rel. Each fusion gene contains at least three Dorsal binding sites within 400 bp. Only six of the 16 fusion genes contain matches to the GATA consensus sequence (summarized in Figure 4A and Table 1), and four of these contain tightly linked REL and GATA sites: Ady, Fas3, Runt, and Zen. Transgenic larvae containing each of the 16 lacZ fusion genes were tested, and only two exhibited expression in the fat body after infection or injury, Ady and Runt (Figure 4B). Remarkably, the regulatory regions of these two genes contain closely linked REL and GATA

Table 1. Matches to SELEX Consensus Sites within Cluster/lacZ Constructs

	Matches to Rel			Matches to Srp				Matches to Rel			Matches to Srp		
Enhancer	SELEX Consensus			SELEX Consensus			Enhancer	SELEX Consensus			SELEX Consensus		
Attacin A	(−410)	GGGATTATT	(−418)	(−110)	TGATAAGG	(−103)	Ady	(−213)	GGGAAATCC	(−221)	(−69)	CGATAAAT	(−76)[a]
	(−348)	GGGATTCTC	(−356)					(−182)	GGGTTTTCC	(−190)			
	(−123)	GGGAATTTC	(−115)					(−151)	GGGTTTTTC	(−159)			
	(−44)	GGGAAGAAC	(−52)					(−109)	GGGAAAAAC	(−117)			
Cecropin A1	(−220)	GGGAACAAT	(−228)	(−136)	AGATAAGG	(−143)	CG9595	(−1234)	GGGTCAACG	(−1226)	(−1089)	TGATAAGT	(−1082)
	(−152)	GGGATTTTT	(−160)					(−1213)	GGGTTAAAG	(−1221)	(−963)	AGATAGGT	(−970)
Diptericin	(−148)	GGGATTCCT	(−140)	(−68)	AGATAAGG	(−61)		(−1191)	GGGAACCCT	(−1199)			
	(−52)	GGGATTCCT	(−44)					(−995)	GGGTTAACT	(−987)			
Drosomycin	(−662)	GGGTTTTTC	(−654)	(−613)	CGATAGGC	(−606)		(−871)	GGGATTAACCC	(−861)*			
	(−481)	GGGATACAG	(−489)	(−497)	AGATAAGC	(−504)		(−680)	GGGTTTTCCC	(−671)*			
	(−319)	GGGTTTAAC	(−311)	(−353)	TGATAACA	(−346)		(−565)	GGGAAAATCCC	(−555)*			
	(−148)	GGGAACTAC	(−156)	(−170)	CGATAAGC	(−163)		(−307)	GGGAAAAAC	(−299)			
Metchnikowin	(−330)	GGGTAATTT	(−322)	(−201)	TGATAATC	(−194)	Fas3	(−1222)	GGGAAATCC	(−1230)	(−633)	AGATAATG	(−640)
	(−184)	GGGAAGTCCC	(−175)*	(−129)	AGATAACC	(−136)		(−595)	GGGTATTCCC	(−586)*	(−475)	TGATAGCT	(−468)
	(−149)	GGGAACCCA	(−157)	(−85)	CGATAAGC	(−92)		(−426)	GGGAAATCC	(−434)			
	(−62)	GGGACTTTT	(−70)					(−420)	GGGATTTTC	(−412)			
PGRP-SB1	(−439)	GGGATTCAC	(−447)	(−459)	AGATAAGA	(−466)	Phm	(−435)	GGGATTACC	(−427)	(−469)	CGATAAGC	(−476)
				(−366)	AGATAAGT	(−359)		(−188)	GGGAAAAAC	(−196)			
Defensin	(−564)	GGGAATCCA	(−572)	(−251)	AGATAAGA	(−258)		(−173)	GGGAAAACCC	(−164)*			
	(−538)	GGGAGTAAT	(−530)				Runt	(−548)	GGGAAAACC	(−556)	(−343)	TGATAGGA	(−350)
	(−185)	GGGAGAAAC	(−193)					(−529)	GGGATTTCC	(−537)	(−247)	TGATAATT	(−254)
	(−98)	GGGAGTCCC	(−90)*					(−367)	GGGTAAATC	(−375)	(−191)	AGATAAGT	(−198)
Lipase	(−342)	GGGAATTTT	(−334)		N/A			(−225)	GGGTTTTCC	(−233)	(−179)	CGATAACA	(−172)
								(−147)	GGGAAATCC	(−139)	(−46)	TGATAAGC	(−53)
								(−74)	GGGTTACTC	(−82)			
							Zen VRE	(−633)	GGGAAAACC	(−641)	(−474)	CGATAAGA	(−481)
								(−486)	GGGTTTCAG	(−494)	(−216)	TGATAAGT	(−223)
								(−369)	GGGAAATCC	(−361)	(−132)	AGATAATT	(−125)
								(−320)	GGGTTTCTCCC	(−310)*			
								(−251)	GGGAAAACC	(−243)			

An asterisk (*) denotes a palindromic site.

[a]Contains an inexact match to the SELEX consensus.

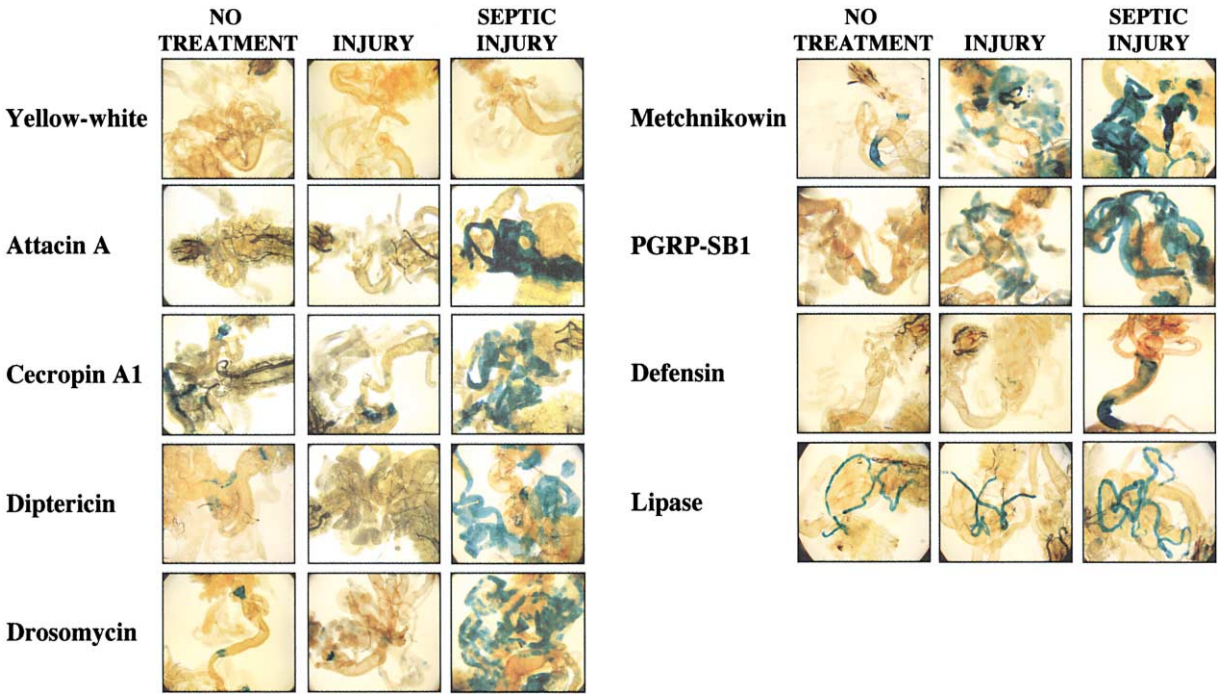


Figure 3. Dissected Transgenic Larvae Are Assayed for lacZ Expression

Wandering transgenic third instar larvae were either poked with a needle dipped in a cocktail of gram-positive and gram-negative bacteria, poked with a clean needle, or left untreated. After 3–6 hr the animals were dissected, fixed in glutaraldehyde, and stained in a solution containing X-gal. Attacin A, Cecropin A1, Diptericin, Drosomycin, Metchnikowin, and PGRP-SB1 enhancers induce robust lacZ expression in the fat body upon septic injury. Injury induces reporter expression to varying degrees, but the levels are almost always lower compared to the infected animals. Larvae bearing these transgenes also produce a constitutive band of lacZ expression around the proventriculus and in a small band around the gut. The Defensin enhancer induces lacZ within the gut upon septic injury and the Lipase enhancer is constitutively active in the Malpighian tubules.

sites positioned in the same orientation. The only other genes that possess a similar organization, Fas3 and Zen, failed to mediate expression in the fat body. However, in both cases, the closest REL and GATA sites map more than 400 bp from the transcription start site. In contrast, the linked sites in the Ady and Runt 5′ regulatory regions map immediately 5′ of the minimal eve promoter in the lacZ expression vector.

Importance of Individual REL and GATA Binding Sites

The activities of the REL and GATA sites were examined in the regulatory DNA of Metchnikowin (Mtk) by transiently transfecting a variety of luciferase (LUC) reporter genes in mbn-2 blood cells (Figure 5). The 345 bp Mtk regulatory DNA contains four predicted REL sites and three GATA sites. It mediates robust induction of the LUC reporter gene upon addition of lipopolysaccharide (LPS) (Figure 5A). Deletion of the distal-most REL site results in a decrease in activity (37.2-fold to 18.9-fold). Two additional deletions, each encompassing a REL/GATA pair, resulted in dramatic reductions in LUC activity. The smallest fusion gene, containing a single pair of linked REL and GATA sites, is insufficient for activation by LPS.

Additional manipulations were done in the context of the 221 bp ΔI fusion gene, which is the smallest Mtk regulatory DNA that mediates strong induction by LPS (Figure 5B). Each of the three GATA sites is essential for optimal expression. However, sites that map close to the transcription start site are more important than those located in more distal regions. For example, mutation of the distal-most GATA site (GATA1) results in a modest decrease in activity, whereas mutation of the proximal GATA3 site results in an ~30-fold reduction in expression. The relative importance of each GATA site also correlates with its in vitro binding affinity for the Serpent protein, with the GATA3 site exhibiting the highest affinity (Figure 5C). Gel shift assays were used to demonstrate that point mutations in the GATA (and REL) sites eliminate binding by Serpent and Relish.

Each of the three REL sites in the 221 bp Mtk regulatory DNA is also essential for optimal expression, although the REL1 and REL3 sites appear to be more critical than REL2 (Figure 5B). Again, there is a correlation between DNA binding affinity and the relative contribution of the site to the induction of LUC expression by LPS (Figure 5C). Strikingly, mutations in all three GATA sites (GATA123 mut) or all three REL sites (REL123 mut) completely abolish LPS induction. This demonstrates the importance of REL-GATA synergy for gene expression. Both sites are required for activation, and even three closely linked REL sites (or GATA sites) are unable to mediate expression.

Systematic mutations in each of the six REL and GATA sites identify the two proximal sites, GATA3 and REL3,

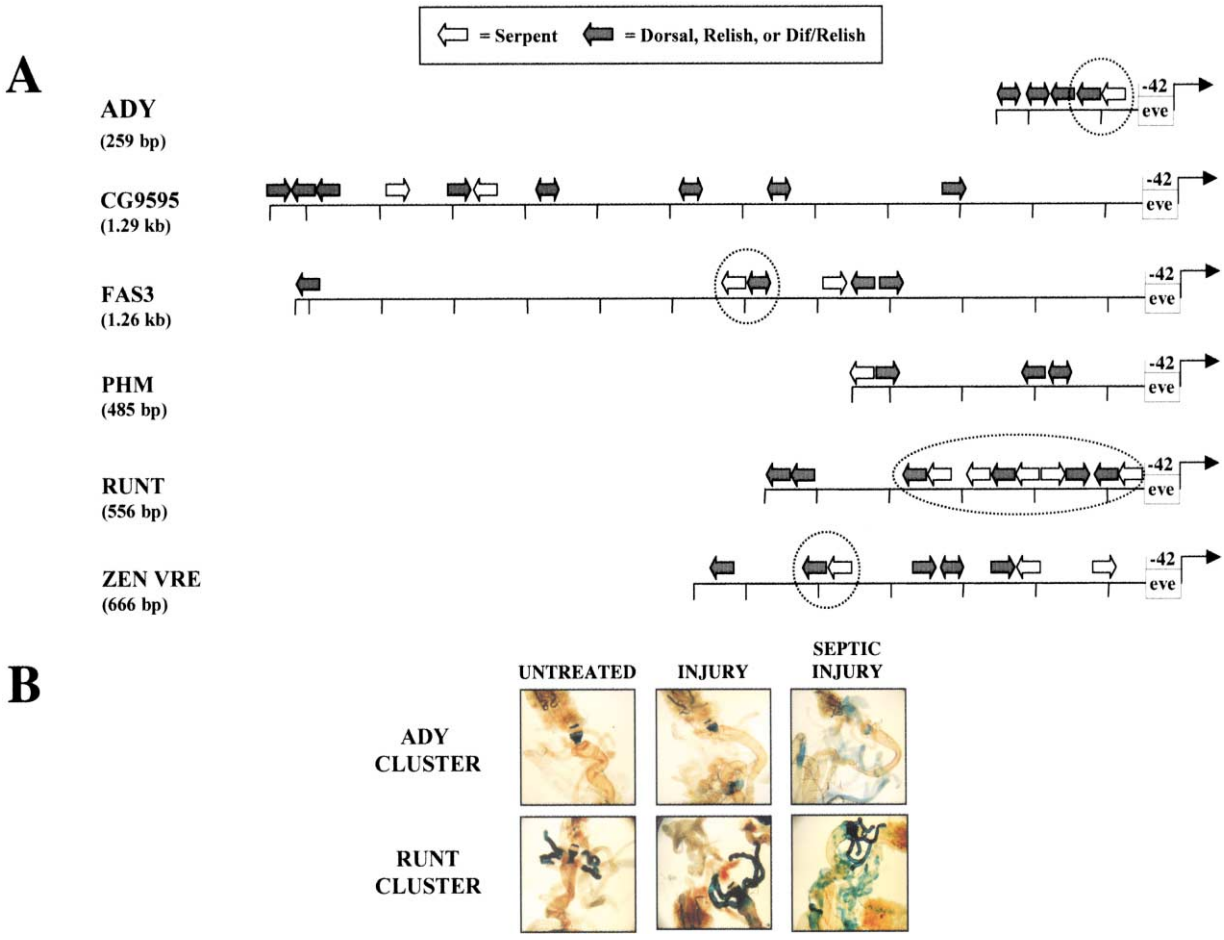


Figure 4. The Response of High-Density Dorsal Clusters to Infection Was Examined

The fly genome contains 16 regions with at least three high-affinity Dorsal binding sites (as found in the zen VRE: GGGWWWWCCM or GGGWDWWWCCM) within 400 bp (Markstein et al., 2002).

(A) Diagrams of 6/16 clusters that contain consensus Serpent sites. Each cluster is named after the gene most proximal to it in the genome. Four clusters contain at least one REL site within 50 bp of a GATA site and in the same orientation (circled within Ady, Fas3, Runt, and Zen). Three independent transgenic lines of all 16 clusters were tested in infection assays.

(B) Two clusters, associated with Ady and Runt, yield infection-induced lacZ expression in the fat body. The Runt cluster also drives constitutive expression in the proventriculus and the four gastric caecae.

as being particularly important for the LPS-mediated induction of LUC expression. Mutations in either GATA3 or REL3 cause a 30- or 61-fold reduction in expression, respectively (Figure 5B). These sites are arranged in the same orientation, but inverting either site (GATA3 inv or REL3 inv) causes a consistent 2- or 3-fold reduction in expression. In these experiments, the other two pairs of REL and GATA sites are not altered. Thus, the orientation of the REL and GATA sites is important for the optimal activation of the minimal Mtk regulatory DNA (see below).

REL-GATA Orientation Is Essential in Transgenic Larvae

Additional studies were done to examine the orientation of linked REL and GATA sites. These assays employed transgenic larvae since this is a more stringent test than the transient transfections used to analyze Mtk expression. The CecA1 regulatory DNA was used since it contains just two REL sites and one GATA site (Figure 6A). The linked sites are positioned in the same orientation, but they are arranged in the opposite order as the REL3 and GATA3 sites in the Mtk regulatory DNA (see Figure 5). Transgenic larvae containing the normal CecA1/lacZ fusion gene exhibit intense β-galactosidase activity in the fat bodies after infection (Figure 6A). However, inversion of the GATA site essentially abolishes this expression. There is some variability in different experiments, but inverting the orientation of the GATA site results in both diminished levels of expression and a reduction in the number of infected larvae that display any staining in the fat body. There is a consistent and reproducible reduction in the activation of the modified CecA1/lacZ fusion gene (see figure legend for a detailed discussion of the number of transgenic strains that were examined). Normal expression was not restored by inverting both the REL and GATA binding sites (see Figure 6A), even though the modified sites are now organized in the same configuration as those seen in the Attacin regulatory

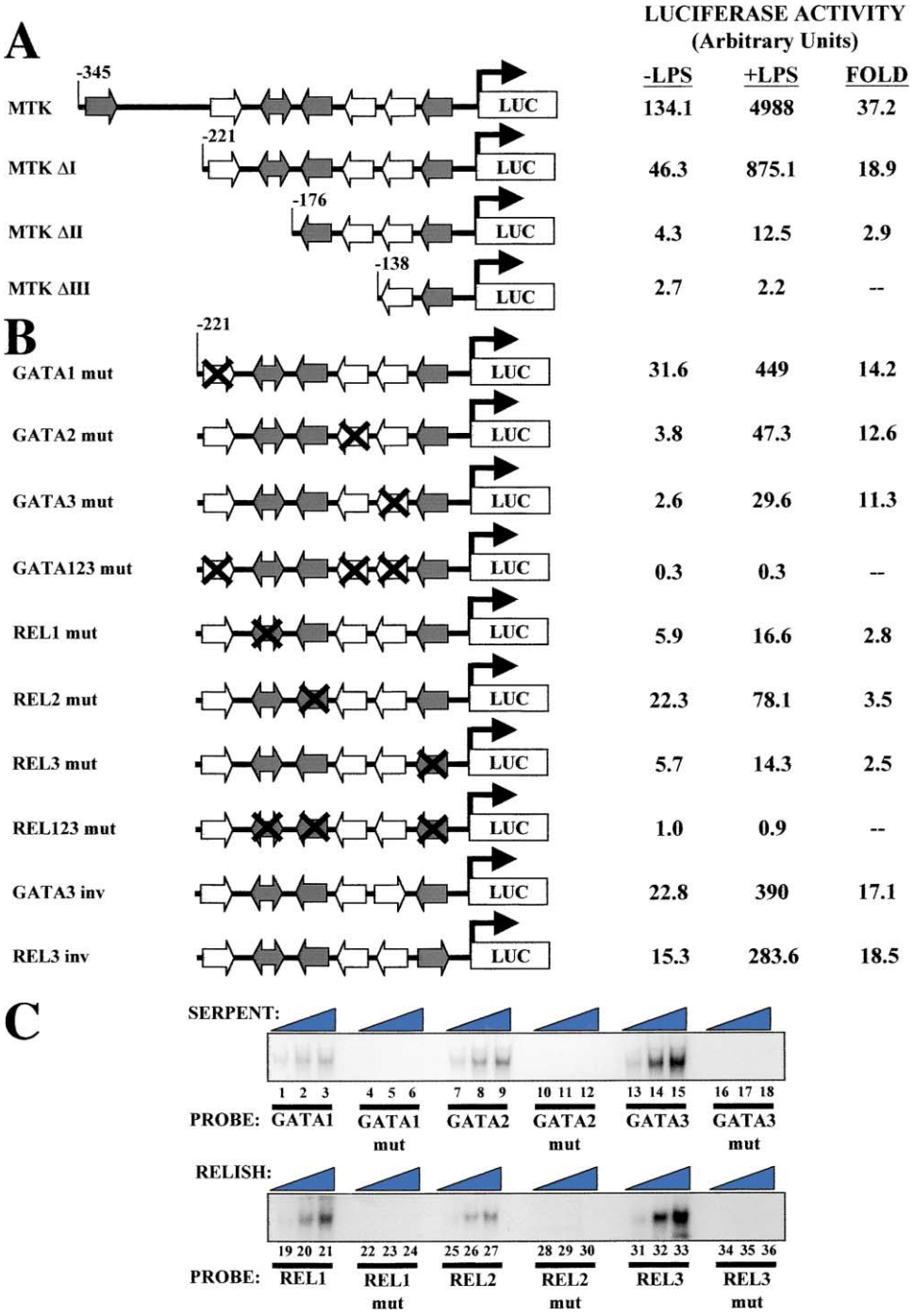


Figure 5. Transient Transfection Assays

The values represent an average of three independent experiments with less than 15% variability among them.

(A) The 345 bp Metchnikowin enhancer was placed upstream of a luciferase reporter (LUC) and transfected into the mbn-2 cell line. Upon incubation with LPS for 9 hr, the enhancer is strongly induced 37.2-fold. Successive deletion of sequences from the 5′ end results in diminished transcription. The minimal inducible construct is a 176 bp fragment containing two REL sites and two GATA sites (ΔII).

(B) Mutation of individual REL and GATA sites within the ΔI construct reveals a differential requirement for each. Mutation of either all three REL sites or all three GATA sites results in no activity, indicating a crucial role for each type of site.

(C) EMSA assays were conducted on radiolabeled oligos bearing normal or mutant binding site sequences. Increasing amounts of recombinant Relish and Serpent proteins were incubated with the oligos, and the resulting complexes were resolved on nondenaturing polyacrylamide gels.

DNA (see Figure 2). The analysis of the Mtk and CecA1 regulatory DNAs suggest that the orientation of linked REL and GATA sites is essential for the induction of immunity gene expression, but there are additional aspects of the regulatory architecture that are also important (see Discussion).

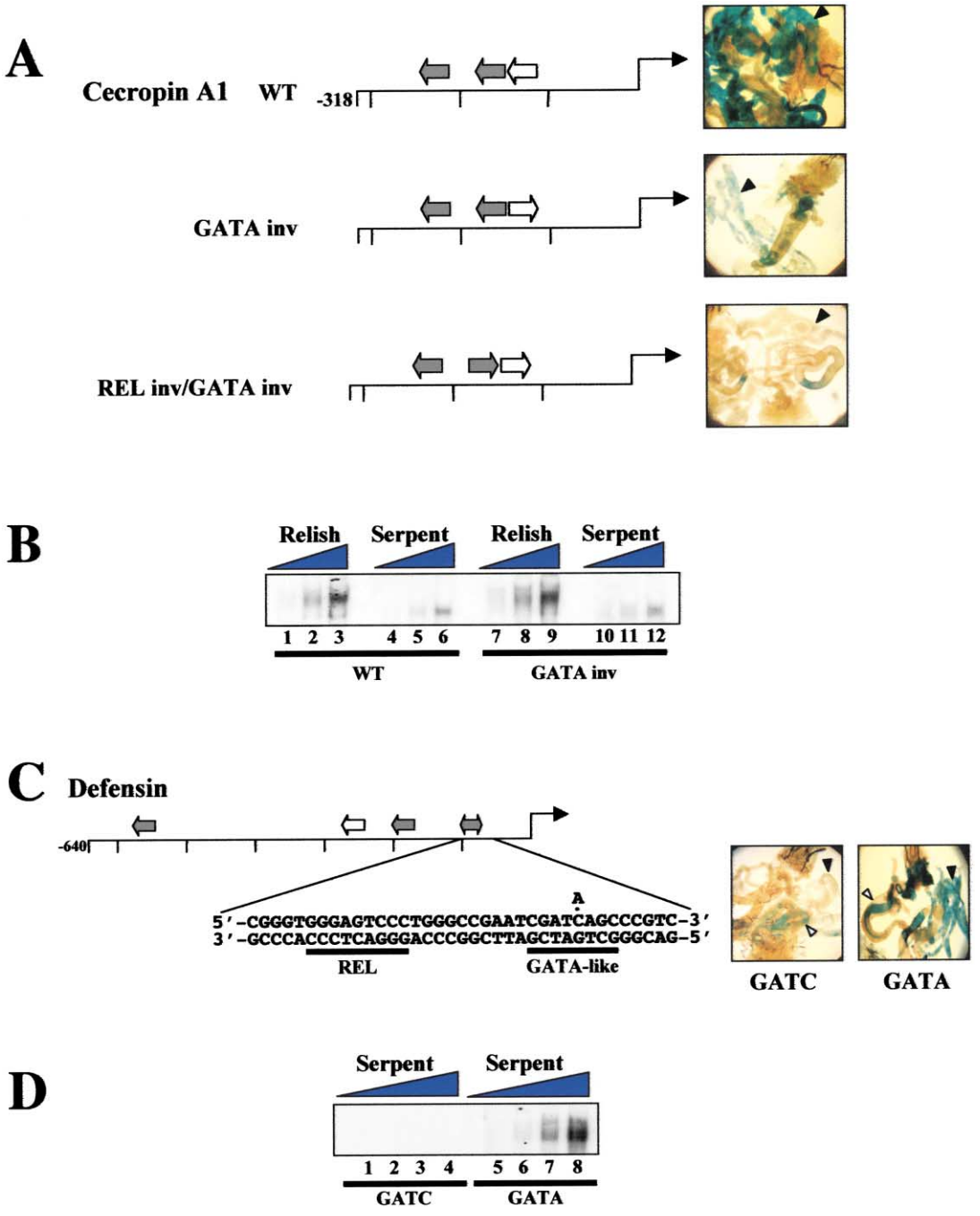


Figure 6. Aspects of Grammar Are Necessary and Sufficient for an Immune Response

(A) The GATA site within the Cecropin regulatory region was inverted. When this enhancer is compared to the unaltered Cecropin regulatory DNA, ~3 times fewer animals express lacZ in the fat body upon infection. Inversion of the REL site to match the GATA site results in no detectable lacZ expression. Four independent lines were analyzed per construct. Out of at least 50 individuals tested, the following percentages expressed the reporter gene: 72% for wild-type Cec A1 (most were robust), 23% for GATA inv (most were weak), and 0% for REL inv/GATA inv.

(B) Reversal of the GATA site does not negatively affect binding of Relish or Serpent to this region, as shown by EMSA (compare lanes 1–3 with 7–9, lanes 4–6 with 10–12).

(C) Creation of an optimal GATA site in the Defensin enhancer creates a gain of function. Within the Defensin enhancer is a palindromic Rel site adjacent to a sequence reminiscent of a Serpent binding site. A single nucleotide substitution (C→A) converts this sequence into a consensus Serpent site. As a result, the enhancer, which normally induces intestine-specific lacZ expression in response to infection (white arrowheads) additionally induces expression in the fat body (black arrowheads).

(D) In gel shifts, conversion of the GATA-like sequence into a consensus GATA site results in high-affinity binding by Serpent (compare lanes 1–4 with lanes 5–8).

Reduced induction of the modified CecA1 regulatory DNA with an inverted GATA site is not due to diminished binding of Relish or Serpent. Gel shift assays were done to test this possibility (Figure 6B). Relish and Serpent proteins were mixed with short DNA fragments containing the REL-GATA sequences in the wild-type and modified CecA1 enhancers (Figure 6B, compare lanes 1–6 with 7–12). There is no significant difference in the amount of protein-DNA shifted complexes formed on the two fragments. One way to explain the orientation requirement of the linked REL and GATA sites is the cooperative binding of the Rel and Serpent proteins. However, gel shift assays failed to indicate any evidence of cooperative DNA binding interactions (data not shown).

Creating a Synthetic Immune Response in the Larval Fat Body

The preceding experiments suggest that Dorsal, Dif, and Relish function synergistically with Serpent to activate gene expression in the fat body upon infection. Serpent provides the tissue specificity since it is expressed primarily in fat bodies, while the REL proteins provide responsiveness to infection since they are released from the cytoplasm and enter nuclei only after infection. A critical test of this model is to create an immunity enhancer de novo with synthetic REL and GATA binding sites. Toward this end, we modified the Defensin enhancer (Figure 6C). The normal enhancer mediates weak expression in the gut upon infection. It is not active in the fat body within 6 hr postinfection, presumably due to the absence of closely linked REL and GATA sites (see summary in Figure 2). The proximal region of the Defensin enhancer contains a sequence that is related to GATA (CGATCAG). This sequence does not bind the Serpent protein in gel shift assays (Figure 6D, lanes 1–4). However, a single nucleotide substitution was made that converts the sequence into a strong GATA site (CGA TAAG). This site binds Serpent well (Figure 6D, lanes 5–8), and is situated just 10 bp downstream of a palindromic REL site. The modified Defensin enhancer was attached to the lacZ reporter gene and introduced into transgenic larvae. Upon infection, the modified enhancer directs expression in the fat body and gut, whereas the normal enhancer is expressed primarily in the gut (Figure 6C).

Discussion

We have presented evidence that REL-GATA synergy is essential for the activation of several immunity genes in *Drosophila*. This synergy appears to depend on closely linked REL and GATA binding sites that are organized in the same orientation (summarized in Figure 7). Nearly half of the most strongly activated immunity genes (21 of 50) exhibit this type of organization, and if we extend our analysis to the 134 genes known or predicted to function in immunity from the microarray assay, approximately one-third (45 of 134) show the same pattern. Six of the associated regulatory DNAs were explicitly tested in this analysis. Inverting linked REL and GATA sites attenuates the activities of the Mtk and CecA1 regulatory DNAs. Moreover, the creation of an appropriate GATA site in the Defensin regulatory DNA causes it to become active in the fat body. Thus, these studies suggest that immunity regulatory DNAs share a common organization and raise the possibility that eukaryotic enhancers contain constrained structural features rather than being composed of unordered clusters of DNA binding sites.

Previous studies on immunity enhancers have relied primarily on the use of consensus binding sites for mammalian NF-κB proteins. For example, GGGRAYYYY has been used as a consensus Rel binding site to identify fly immunity regulatory DNAs (Hultmark, 1993). This sequence does not allow one to discriminate the recognition sequences for Dorsal, Dif, Relish, and various heteromeric forms of these proteins. Many immunity genes respond differentially to the Toll (Dorsal and Dif) and PGRP-LC/imd (Relish) pathways (Han and Ip, 1999; De Gregorio et al., 2002), and a gene's bias toward one pathway or another may coincide with their exact Rel-like recognition motifs. The Drosomycin gene, for example, is dependent on the Toll signaling pathway for its induction. The Drosomycin regulatory DNA identified in this study contains four REL sites, three of which closely match the SELEX data for Dorsal, a direct target of the Toll pathway. This study also identified a highly specific Dif/Rel heterodimeric recognition sequence: GGGAW TCMC. In the event of a massive infection by multiple classes of pathogens, Dif/Rel may mediate an S.O.S.-type response by ensuring the activation of regulatory DNAs containing this sequence. In this regard we note that the comprehensive microarray assays conducted by De Gregorio et al. (2002) identified Defensin, Metchnikowin, and PGRP-SB1 as requiring both the Toll and PGRP/imd pathways, and we find that the associated regulatory regions contain close matches to the GGG AWTCMC motif.

The most notable feature of the regulatory regions presented in this study is the occurrence of linked REL and GATA sites positioned with the same relative orientation. There are four possible arrangements of tandem REL and GATA sites, and each possible arrangement, or class, is seen in at least one of the immunity regulatory regions tested in this study (summarized in Figure 7). We imagine classes 1 and 3 are equivalent in terms of their REL-GATA stereochemistry, differing only in their orientation relative to the promoter. Likewise, classes 2 and 4 are similar to each other and stereochemically distinct from classes 1 and 3 (see also Model 1). We speculate that the exact type of REL-GATA composite element might dictate what types of Rel factors are permitted to bind or determine the overall architecture of the Rel-Serpent complex.

The importance of having a GATA site with the same polarity as a REL site was demonstrated by inverting the proximal REL and GATA sites within the Mtk and CecA1 regulatory regions. The most obvious explanation for this reliance on REL-GATA orientation is that Rel factors physically associate with the Serpent protein (Figure 7, Model 1). Assuming Rel and Serpent proteins are not perfectly symmetric, reorienting one site would break an interface formed between the factors. A second reason for the orientation requirement may be the presence of factors flanking the REL-GATA unit (X and Y in Model 2). Reversing one or both sites would disrupt key

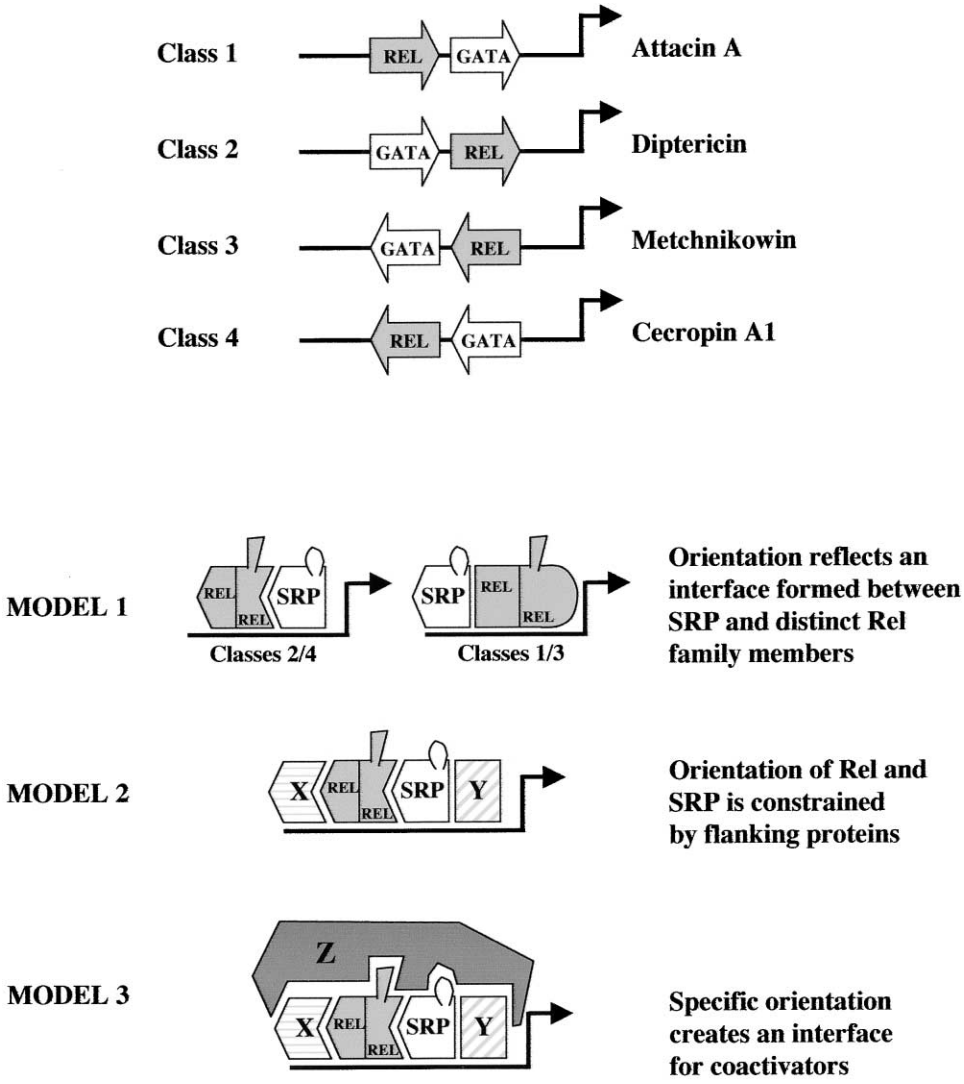


Figure 7. Classes of Binding Site Pairs and Possible Mechanisms of REL-GATA Synergy

contacts with factors bound upstream or downstream. Finally, a properly oriented REL-GATA unit may create a peculiar surface that is recognized by appropriate co-activators (Z in Model 3), such as Mediator or chromatin-modifying complexes (reviewed by Levine and Tjian, 2003). Any of these models would explain why the REL and GATA sites must be in the same orientation, but need not be arranged in the same order. It might also provide an explanation for why inverting both the REL and GATA sites failed to restore the activities of the CecA1 regulatory DNA (see Figure 6A).

The importance of REL-GATA orientation was demonstrated in transgenic larvae. The previous analysis of the mammalian IFN-β enhancer provides a clear precedent for organized binding sites. It contains a binding site for the ATF2/c-Jun heterodimer (PRDIV) adjacent to a site for interferon regulatory factor (IRF) proteins (PRDIII-I). Crosslinking experiments reveal that the orientation of ATF2/c-Jun on the PRDIV site is random in the absence of other factors but becomes fixed in the presence of IRF-3. In addition, only the native orientation of PRDIV allows protein-protein interactions between ATF2 and IRF-3 and is necessary for full activity of the enhancer (Falvo et al., 2000). An open question is whether the configuration of binding sites in *Drosophila* immunity enhancers reflects the formation of an interface between Serpent and a specific Rel subunit (see Figure 7).

There are two extreme views of regulatory DNAs. One holds that they contain loosely organized clusters of binding sites for different transcription factors. Enhancers active early in *Drosophila* development appear to fall into this category. These enhancers are usually 300–500 bp in length and contain on the order of 10 binding sites for several activators and repressors. The eve stripe 2 enhancer, for example, contains multiple binding sites for the Bicoid and Hunchback activators, which function synergistically to activate the enhancer in the anterior half of the early embryo (Small et al., 1992). The enhancer operates normally when a GAL4-Sp1 fusion protein is substituted for Hunchback, thereby demonstrating the lack of dedicated interactions among the stripe 2 activators (Arnosti et al., 1996). Another view

of enhancers is seen for the mammalian IL-2 and IFN-β genes. In both cases, multiple transcription factors bind to a compact region of DNA and exhibit a strict spatial organization. In the case of the IFN-β enhancer, a higher order complex termed the enhanceosome is formed due to multiple cooperative interactions between transcriptional activators and architectural proteins (Thanos and Maniatis, 1995). The net result is the formation of a special surface required to recruit the CBP/PolII coactivator (Merika et al., 1998).

Immunity regulatory DNAs exhibit an organization that is intermediate between fly embryonic enhancers and mammalian enhanceosomes. There appear to be organizational constraints on just a special subset of *cis*-regulatory elements, the REL and GATA sites, so that these proteins can function in a synergistic fashion for the rapid induction of immunity gene expression in response to infection. The combination of microarray assays and bioinformatics methods should permit the identification of any constrained features in the regulatory DNAs underlying other processes such as circadian rhythms and olfaction. The occurrence of a fixed organization of regulatory elements should increase the hit rate of whole-genome searches for coordinately regulated enhancers. The greater the constraint on the organization, the better the prospects for elucidating gene expression networks from simple genome sequence data.

Experimental Procedures

Plasmids

Protein expression constructs for Dorsal, Dif, Relish, and Serpent were generated by cloning the appropriate PCR fragments into the pRSET vector (Invitrogen). Dorsal (aa 1–678), Dif (aa 1–667), and Serpent (aa 1–950) were cloned between NcoI and HindIII sites; Relish (aa 3–595 and aa 113–595) between Kpn I and Eco RI. Full-length GST-Dif was a gift from Tony Ip. Enhancers were PCR amplified from genomic DNA with a 5′ primer containing a BglII site and a 3′ primer containing a BamHI site. The products contained the start codon, 5′ UTR and flanking promoter region of the genes indicated. These were cloned as in-frame fusions to lacZ in a pCaSpeR vector modified by Hilary Ashe to contain SuHw insulators flanking the multiple cloning site and lacZ gene. Mutagenesis was performed using primers harboring the desired mutation flanked by 15–20 nt of sequence. PCR reactions were carried out with pfu polymerase (Stratagene), and the parent vector was digested with DpnI (New England BioLabs) for 2 hr at 37°C. Digests were transformed into DH5α cells, and the presence of the mutation detected by sequencing. Mutations of binding sites in the Metchnikowin enhancer were created by conversion of the 5′ most G in the GATA sites to A, and conversion of the GGG characteristic of every REL site to CCC.

Protein Purification

Proteins were expressed as hexahistidine or GST fusions in *Escherichia coli* BL21(DE3) pLysS, or HB101 bacteria, respectively, using the pRSET and GST expression vectors described above. Bacteria were grown to an optical density at 500 nm of 0.5 to 0.8 at 37°C and then induced with 1 mM IPTG for 3 hr. His-tagged proteins were purified under denaturing conditions and purified by affinity chromatography using Ni-NTA columns (Qiagen) according to the manufacturer's instructions, with the addition of 10 μM ZnSO4 in the binding and washing steps for Serpent. GST-Dif was purified under native conditions. Dif/Relish heterodimers were created by codializing equimolar amounts of His-Dif (F.L.) and His-Relish (aa 3–595 or aa 113–595).

SELEX Assays and EMSAs

For SELEX assays, a radiolabeled oligonucleotide with the sequence 5′-CTCAGGATCCAGTTCAGCGGTCGCTGATC-(N_{25})-GATCCGAATTCAGTGCAACTGCAGC-3′ (Operon Technologies) was incubated with 200 ng/μg of recombinant protein in a solution of 10 mM Tris (pH 7.5), 50 mM NaCl, 5% glycerol, 5% sucrose, 0.2 mM EDTA, 7.4 mM $MgCl_2$, 0.5 μg BSA, 5 mM DTT, 0.1% NP-40, and 0.5 μg pdI:dC. Protein-DNA complexes were resolved by electrophoresis through a 5% acrylamide:bisacrylamide (29:1) gel in 0.5× TBE (40 mM Tris, 45 mM Boric acid, and 1 mM EDTA) at 200 V for 3 hr. Complexes were detected by autoradiography, excised, and eluted overnight in 0.5 M NH_4OAc and 1 mM EDTA. The eluted DNA was precipitated and PCR amplified with primers flanking the 25 nt N-mer and radioactive nucleotides. PCR products were purified on a G50 column (Sigma) and cleaned with phenol:chloroform. This process was repeated four times. The final PCR products were cut with BamHI and EcoRI (New England BioLabs) and cloned into a vector for sequencing. At least 30 sequences were collected for the consensus binding sites. The probe sequences for Figure 5 were synthesized by Operon Technologies and are as follows: Mtk GATA1 (5′-TAGGCT GATAATCCGG-3′), GATA1mut (5′-TAGGCTAATAATCCGG-3′), GATA2 (5′-GGGGCAGATAACCGAT-3′), GATA2mut (5′-GGGGCAAATAACC GAT-3′), GATA3 (5′-TTCTGCGATAAGCAGC-3′), GATA3mut (5′-TTC TGCAATAAGCAGC-3′), REL1 (5′-ACCGTGGGAAGTCCCCTTTG-3′), REL1mut (5′-ACCGTCCCAAGTCCCCTTTG-3′), REL2 (5′-TGGCT GGGTTCCCCTGGC-3′), REL2mut (5′-TGGCTCCCTTCCCCTGGC-3′), REL3 (5′-GATTGGGGACTTTTTATTT-3′), REL3mut (5′-GATTGCCCA CTTTTTATTT-3′). The probe sequences for Figure 6 are as follows: CecA1 WT (5′-TGTACTTTTCTCTGCAAAAATCCCCGTGCATGCCTT ATCTGTCAT-3′), CecA1 GATA flip (5′-TGTACTTTTCTCTGCAAAAA TCCCCGTGCAAGATAAGGCAGTCAT-3′), Def WT (5′-GATCCGGG TGGGAGTCCCTGGGCCGAATCGATCAGCCCGTC-3′), Def GATA new (5′-GATCCGGGTGGGAGTCCCTGGGCCGAATCGATAAGCCC GTC-3′). One hundred picomoles of double-stranded oligos was labeled with 6 μl of [γ-^{32}P]ATP (NEN) and 1 μl of polynucleotide kinase in 1× PNK buffer (New England BioLabs) for 1 hr at 37°C. The labeled DNA was purified on a G50 column as before. Binding reactions were performed essentially as described above.

Infection and β-Gal Staining

Transgenic climbing third-instar larvae were poked with a pulled glass capillary (Frederick Haer & Co) dipped in a pelleted mixture of *Micrococcus luteus* and *Escherichia coli* bacteria. As controls, larvae were poked with clean capillaries or left untreated. The animals were allowed to recover on apple juice agar plates at room temperature for 3–6 hr. After infection larvae were dissected in *Drosophila* Ringer's solution (182 mM KCl, 46 mM NaCl, 3 mM $CaCl_2$, 10 mM Tris-HCl 8.0) and fixed (1% glutaraldehyde, 50 mM sodium cacodylate) for 15 min at 37°C. The entrails were briefly rinsed with prestaining buffer (7.2 mM Na_2HPO_4, 2.8 mM NaH_2PO_4, 1 mM $MgCl_2$, 0.15 M NaCl) and incubated in staining solution (prestain plus 5 mM $K_4[Fe(CN)_6]$, 5 mM $K_3[Fe(CN)_6]$, 0.2% X-gal) until lacZ expression became visible (15 min to 3 hr). Dissected larvae were mounted in a solution of 50% glycerol/50% 1× PBS. At least three independent transgenic lines were tested in every case.

Cell Culture and Transient Transfections

mbn-2 cells were maintained at 25°C in Schneider's Insect Media (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Sigma) and 1 × Pen/Strep (Sigma). For transfections, cells were seeded in 24-well plates at a density of 2×10^6 cells/ml. The day after plating, transfections were carried out using the calcium phosphate method (Rio and Rubin, 1985). The following amounts of DNA were used: 0.1 μg of each reporter per well plus 2 μg of CMVZ, which served as an internal control for transfection efficiency. Two days after transfection, 10 μg of LPS (O127:B8, Sigma) was added, and the cells were harvested 9 hr later. Cell extracts were made with lysis buffer (Promega), and luciferase activity was measured on a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

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