Functional Characterization of a Novel Promoter Element Required for an Innate Immune Response in *Drosophila*

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Innate immune reactions are crucial processes of metazoans to protect the organism against overgrowth of faster replicating microorganisms. *Drosophila melanogaster* is a precious model for genetic and molecular studies of the innate immune system. In response to infection, the concerted action of a battery of antimicrobial peptides ensures efficient killing of the microbes. The induced gene expression relies on translocation of the *Drosophila* Rel transcription factors Relish, Dif, and Dorsal to the nucleus where they bind to κ B-like motifs in the promoters of the inducible genes. We have identified another putative promoter element, called region 1 (R1), in a number of antimicrobial peptide genes. Site-directed mutagenesis of the R1 site diminished *Cecropin A1* (*CecA1*) expression in transgenic *Drosophila* larvae and flies. Infection of flies induced a nuclear R1-binding activity that was unrelated to the κ B-binding activity in the same extracts. Although the R1 motif was required for Rel protein-mediated *CecA1* expression in cotransfection experiments, our data argue against it being a direct target for the *Drosophila* Rel proteins. We propose that the R1 and κ B motifs are targets for distinct regulatory complexes that act in concert to promote high levels of antimicrobial peptide gene expression in response to infection.

The innate immune response is activated as a first line of defense against infections by different classes of microorganisms (14, 21). In *Drosophila melanogaster*, the early phase is manifested by the inducible expression of an array of antimicrobial peptides to combat the infection (1, 3). This inducible expression relies on the activity of the *Drosophila* Rel transcription factors Dorsal, Dif, and Relish (7, 17, 36). In response to an infection, these factors translocate from the cytoplasm to the nucleus where they bind to promoter elements referred to as κ B or κ B-like motifs (10). In recent years, work from several groups have shown that the *Drosophila* Rel factors are activated in response to at least two independent signaling pathways, the Imd/Relish pathway responding primarily to infection by gram-negative bacteria and the Toll/Dif pathway responding mainly to infection by fungi and gram-positive bacteria (16, 25, 41). Although both pathways can activate all three *Drosophila* Rel factors, Relish, Dif, and Dorsal, it has been shown that flies with mutations in the *Relish* gene are especially susceptible to infection by gram-negative bacteria (13). Likewise, flies lacking a functional copy of the *Dif* gene are sensitive to fungal infections (32).

The expression of the genes for the antimicrobial peptides CecropinA1 and A2 (*CecA*) is activated by both gram-negative and gram-positive bacteria as well as by fungi (18, 25, 34). This suggests that both the Toll/Dif and the Imd/Relish pathways are contributing to *CecA* gene activation. Experimental data showing that *CecA* expression is reduced, but not abolished, in

flies lacking a functional copy of Relish supports this (6, 13). Similarly, the expression of the *Cec* genes is reduced but not eliminated in mutants lacking a functional copy of the *Dif* gene (32). If both pathways are interrupted, there is very little expression of the *CecA* genes (6, 25). This suggests that independent *cis*-acting elements are targets for these Rel factors in the *CecA* promoters. In contrast, induction of *Diptericin* expression in response to lipopolysaccharide (LPS) was demonstrated to require two copies of an identical κ B motif (dipt- κ B) (20). This dipt- κ B motif is most likely a direct target of the Relish transcription factor, since the presence of a dipt- κ B binding activity present in wild-type (WT), bacterium-challenged flies was absent in *Relish* mutant flies (33). In addition, the expression of the *diptericin* gene is nearly abolished in *Relish* mutant flies (13) but is present at normal levels in *Dif* and *Dorsal* mutant flies (32).

We have previously shown that 112 bp of upstream sequence of the *CecA1* gene is sufficient for LPS-inducible reporter gene expression in cell culture transfections and in transgenic flies (31). This short upstream sequence contains one κ B motif and one GATA motif, both involved in normal expression of the *CecA1* gene (10, 19). The GATA site is primarily required for systemic expression in hemocytes and fat body while the κ B motif was found to be necessary in all tissues analyzed, including barrier epithelia (28, 30, 31, 39). We noticed, however, that the 112-bp 5' region also contains another sequence that is conserved between the *CecA1*, *CecA2*, and *CecB* genes. This sequence, which we tentatively called region 1 (R1), is located in close proximity to the κ B site (9). When a 40-bp fragment, containing both the κ B and the R1 motifs, was deleted from the *CecA1* upstream region, this promoter did not respond to signaling, as demonstrated by the lack of inducible *CecA-lacZ* expression both in cell culture transfections and in transgenic

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larvae and flies (31). In the present study, we investigated the relative importance of the R1 and κ B motifs for the activation of the *CecA* genes and explored the possibility that these two sites respond differently to the two known signaling pathways.

This study demonstrates that the R1 sequence constitutes a novel *cis*-acting motif that is required for normal expression of the *CecA1* gene in the fat body of larvae and flies and in a cell line of hemocytic origin. Although the R1 motif resembles the κ B sequence and its presence is required for Rel protein-mediated expression, our data argue against it being a direct target for any of the three *Drosophila* Rel proteins but instead for another unrelated DNA-binding activity.

MATERIALS AND METHODS

Recombinant DNA. The construction of the *CecA1-lacZ* plasmids pA10 and pA15 has been described previously (10, 29). Plasmids pA18 and pA19 were constructed by site-directed mutagenesis of pA10 by a PCR-based strategy (15). This introduced base substitutions in the R1 and κ B sequences. The substitutions are the same as in the mutant oligonucleotides used in the electrophoretic mobility shift assays (EMSAs). Constructs with 5- and 10-bp spacer sequences inserted between the R1 and κ B sites in pA10 were created with the QuikChange site-directed mutagenesis kit (Stratagene). For P-element transformations, the *XhoI-XbaI* fragment of the pA18 and pA19 constructs were moved from the pBSlac20 vector to the P-element vector pW8 (22). All constructs were sequenced to verify the mutations and the integrity of the remaining upstream region.

P-element-mediated transformation. Transformation was done according to the method of Spradling, except that the recipient strain was *yw* (35). Plasmids pWA18 and pWA19 were injected together with the Δ 2-3 helper plasmid (24). The enclosed GO flies of four independent insertions of pW18 and six independent insertions of pWA19 were individually crossed to establish stable, balanced strains named pA18 a to d and pA19 a to f.

Cell cultures and transfection. *Drosophila* mbn-2 cells (11) were grown at 25°C in Schneider's medium as described previously (29). Cells (5 ml, 1.5×10^6 cells/ml) were plated on 5-cm-diameter dishes and used for transfection by the calcium phosphate precipitation method according to reference 10, except that the cells were harvested at day 4 of transfection. For each transfection, 1 μ g of *CecA1-lacZ* reporter plasmid (pA10, pA15, pA18, and pA19), 100 ng of pActCAT plasmid, and 250 ng of empty expression plasmid pAct5CPL were mixed with carrier plasmid DNA to a total amount of 20 μ g of DNA per transfection. In cotransfection experiments, 250 ng of pAct-Dif, pAct5C-dl, pAct-Relish, or the empty pAct5CPL vector was coprecipitated with the reporter plasmids. For expression of tagged versions of the Rel proteins under the control of the Act5C promoter, transfection was carried out with 0.5 μ g of pActRelish-FLAG, pActDif-V5, or pActdl-V5 (12; A. Wickberg, unpublished data) together with carrier DNA to a total of 20 μ g of total DNA. The cells were incubated with the precipitates for 16 to 24 h at 25°C, washed carefully with serum-free medium, fed with complete Schneider's medium, and incubated at 25°C for 72 h. An immune response was activated by the addition of purified LPS (10 μ g/ml) 4 h prior to harvest. Extraction and reporter gene assays were done as described in reference 10, except that the expression of the transfection efficiency control, pActCAT, was measured by using the CATELISA kit (Boehringer Mannheim).

Infection. Bacteria (*Micrococcus luteus* and *Enterobacter cloacae*) were grown over night in Luria-Bertani medium. Yeast (*Saccharomyces cerevisiae*) was grown over night in yeast extract-peptone-dextrose medium at 30°C. The overnight cultures were washed once in phosphate-buffered saline (PBS; pH 7.0) and diluted 10 times in PBS before injection. Larvae or flies were infected with one of the bacterial suspensions by using a glass capillary with a microinjector (TriTech Research, Los Angeles, Calif.). The injected larvae and flies were kept under humid conditions at 25°C for the desired time.

Protein extract preparation. Nuclear extracts for EMSA from mbn-2 cells were prepared as follows. Confluent cells (5 ml) were harvested, pelleted, and washed once in PBS. The pellet was resuspended in 100 μ l of buffer 1 (10 mM HEPES [pH 7.9], 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol [DTT], and freshly made protease inhibitor cocktail [Boehringer Mannheim]). The homogenate was chilled on ice for approximately 15 min, and the cells were lysed by the addition of 0.6% (vol/vol) Nonidet P-40 followed by repeated inversions of the tube. The nuclei were pelleted at maximal speed for 10 min in a microcentrifuge, and the supernatant, containing cytosolic proteins,

was transferred to a new tube. Fifty microliters of ice-cold buffer 2 (20 mM HEPES [pH 7.9], 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10% [vol/vol] glycerol, and protease inhibitors [Boehringer Mannheim]) was added to the nuclei, and the pellet was resuspended with a wide-bore tip. The nuclei were lysed by vigorous shaking at 4°C for 30 min. Debris was pelleted at maximal speed for 10 min in a microcentrifuge, and the supernatant, containing nuclear proteins, was transferred to a new tube. Nuclear extracts from flies were prepared by gently homogenizing 30 to 40 flies in 250 μ l of buffer 1 and proceeding as described above.

Total extracts from mbn-2 cells, previously transfected with pActRelish-FLAG, pActDif-V5, or pActDorsal-V5, were prepared as follows. Confluent cells (5 ml) were harvested, pelleted, and washed once in PBS. The pellet was resuspended in 500 μ l of PBS and transferred to a microcentrifuge tube, pelleted, and resuspended in 3 volumes of buffer C (20 mM HEPES [pH 7.9], 0.56 M KCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 2 mM DTT, 25% glycerol, and protease inhibitors [Boehringer Mannheim]). The cells were frozen in liquid nitrogen, thawed on ice, homogenized by pipetting, and left on ice for 45 min. The homogenization was then repeated, the debris was pelleted at maximal speed in a microcentrifuge for 10 min, and the supernatant was transferred to a new tube. The protein concentrations of all extracts were measured by the Bradford assay (Pierce), and the extracts were stored at -80°C.

β -Gal assays with larvae and flies. Larvae and flies were injected with a mixture of overnight cultures of *E. cloacae* and *M. luteus* and incubated at 25°C for 4 h to mount an immune response. Larvae were dissected, fixed, and stained for β -galactosidase (β -Gal) activity in situ, with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) as a substrate, as described in reference 31. For quantitative measurements of β -Gal activity, 20 control or bacterium-injected flies were decapitated, grained with a mortar on dry ice, suspended in 75 μ l of buffer C, and prepared as described for total extracts of mbn-2 cells (see above). Spectrophotometric measurements of β -Gal activity were done according to the method of Miller (27) with *o*-nitrophenyl- β -D-galactopyranoside as a substrate, and protein concentrations were determined by the Bradford protein assay (Pierce). The relative β -Gal activity was calculated per protein content in each sample.

EMSA. Deoxynucleotides were labeled with [α -³²P]dCTP (Amersham) and Klenow fragment of DNA polymerase (Life Technologies). The oligonucleotides used were as follows: R1 wt, 5'-d(tcgaactcaGTGTACTTTTctctga); R1 mut, 5'-d(tcgaactcaCTGTAAATAAGctctga); κ B wt, 5'-d(tcgaacacGGGGATTTTgcac); κ B mut, 5'-d(tcgaacacGTTGATTGGTgcac). Capital letters refer to the consensus sequence of the R1 and κ B sites in *Drosophila* immune genes. Underlined bases indicate the altered nucleotides in the mutant probes. Binding reactions were carried out by mixing labeled (approximately 50,000 cpm per reaction) and unlabeled (for competition experiments) probes with 10 μ g of nuclear extract from mbn-2 cells, 10 μ g of total extract from transfected mbn-2 cells, or 40 μ g of nuclear extract from flies, 60 μ g of bovine serum albumin, and 3 μ g of poly(dI-dC) in 20 μ l of EMSA buffer (100 mM NaCl, 15 mM HEPES [pH 7.5], 0.75 mM EDTA, 1 mM DTT, and 8% glycerol). After incubation at room temperature for 15 min, 10 $\times$ loading buffer was added and adjusted with TE (0.1 M Tris [pH 7.8], 1 mM EDTA) to give a final concentration of 5% glycerol and 0.025% bromophenol blue. The samples were loaded on a 5% native polyacrylamide gel. The gel was prerun at 100 V for 45 min at room temperature in TBE (90 mM Tris-borate [pH 7.8], 2 mM EDTA) before the samples were loaded. Electrophoresis was carried out at 200 V for 90 min in TBE at room temperature. The gel was dried, exposed to a phosphor screen, and scanned with a PhosphorImager (Molecular Dynamics).

Extracts treated with antiserum were preincubated with 1 μ l of the respective antiserum for 10 min at room temperature prior to the addition of the probe. The antibodies used were rabbit anti-Relish (37), rabbit anti-Dif (4), monoclonal mouse anti-Dorsal (a generous gift from R. Steward), rabbit anti-FLAG (Sigma), and rabbit anti-V5 (Invitrogen).

RESULTS

The upstream region of the *Drosophila Cec* genes contains a 40-bp region with a high degree of sequence homology (9, 40). Three sequence motifs have been identified within these 40 bp; the κ B-like, the GATA, and the R1 motifs (9). As previously reported, a large number of genes encoding antimicrobial peptides in insects contain clusters of κ B and GATA sites (19). We did a similar search for clustered κ B and R1 sites and found that the *Drosophila Defensin* gene contains such a cluster, as do three of the *Cecropin* genes of *Drosophila virilis* (42) (Fig. 1A).

FIG. 1. (A) Nested R1 and cecκB sequences found in the 5' region of *Cecropin* and *Defensin* genes. Capital letters indicate conserved nucleotides. Numbers refer to positions relative to the transcriptional start site. (B) The RBA is specific for the R1 sequence. EMSA with nuclear extracts from LPS-treated *Drosophila* mbn-2 cells in the absence (–) or presence of increasing concentrations of competing oligonucleotides. For sequences, see Materials and Methods.

The *Defensin* and *Cecropin* peptides are functionally related in that they possess strong activity against gram-positive bacteria (23). The *Cecropins* are also active against gram-negative bacteria and fungi (8, 23). We decided to investigate the function of the κB-R1 element in the promoters of these genes and the relative importance of these two sites in the response to infection.

It has previously been shown that a κB-binding activity is induced in nuclei of mbn-2 cells after challenge with bacteria or LPS (10). As shown by EMSA, a specific R1-binding activity is present in nuclei of mbn-2 cells after treatment with LPS (Fig. 1B) as well as after treatment with gram-negative and gram-positive bacteria and peptidoglycan (data not shown).

Nonspecific binding of nuclear proteins to the ³²P-labeled R1 probe was ruled out by carrying out competition experiments (Fig. 1B).

A comparison of the R1 and the κB sequences shows that there is significant homology between them in the 5'-to-3' direction on opposite strands (Fig. 1A). We therefore tested whether they would be targets for similar or even identical DNA-binding complexes. For clarity reasons, we will refer to the κB sequence used in this study as the cecκB motif. EMSAs with nuclear extracts from the *Drosophila* mbn-2 cells revealed differently migrating DNA-protein complexes with the R1 and the cecκB probes (Fig. 2). In addition, the R1-binding activity (RBA) was effectively competed by increasing the concentra-

FIG. 2. The RBA and κ BA display different binding specificities and do not interact with the same motifs. EMSA with nuclear extracts from *mbn-2* cells incubated with 32 P-labeled R1 probe (A) or *cecκB* probe (B) in the absence (–) or presence of increasing concentrations of unlabeled R1 or *cecκB* oligonucleotides.

tion of unlabeled R1 probe but not *cecκB* probe (Fig. 2A), whereas the κ BA was effectively competed by the *cecκB* probe but not by the R1 probe (Fig. 2B). We conclude that the composition of the complexes interacting with the R1 and *cecκB* sequence motifs in *mbn-2* cells is different and that both types of complexes exist independently of one another.

To investigate the function and relative contribution of the R1 and the *cecκB* sites for inducible expression *in vivo*, we created *CecA1-lacZ* promoter-reporter constructs with independent mutations in the R1 and *cecκB* sites (Fig. 3). The *CecA1-lacZ* constructs with mutated R1 (pA18) and *cecκB* (pA19) sequences were used to create transgenic flies. More than 40 larvae of each transformant strain were analyzed before and after infection with bacteria. Transgenic larvae carrying the pA10 construct conferred strong β -Gal staining in the

fat body in response to bacterial injections in all infected larvae while uninfected larvae did not confer any reporter gene expression (compare Fig. 4A and B). Removal of both elements led to a complete loss of reporter gene activity (Fig. 4C). Destruction of either the R1 or the κ B site severely reduced the expression from the *CecA1* promoter in the fat body after bacterial infection (Fig. 4D and E). Analyses of larvae of four independent transformant pA18 lines revealed that three lines did not confer any significant levels of reporter gene expression (Fig. 4D) while one of the A18 lines (A18c) showed a low but significant level of expression in the fat body after bacterial infection (data not shown), but it was considerably weaker than that in A10, however. Six independent A19 lines were analyzed, and none showed bacterium-inducible reporter gene expression in the larval fat body. Several A19 lines revealed constitutive tissue-specific expression in nonimmune tissues, and two lines conferred weak expression in the fat body, but this expression was not influenced by the injection of bacteria. Thus, inducible *Cec* expression in response to bacterial infection required both the R1 and κ B sites. The results were confirmed by quantitative measurements of the reporter gene activity in extracts of adults (Fig. 4F). Adults of three independent transgenic strains of both A18 and A19 were analyzed. Mutations in either the R1 or the *cecκB* site hampered the reporter gene expression to a level below 30% of that of the WT promoter in extracts of all transformants (Fig. 4F). Only one of the A18 lines conferred weak levels of inducible expression (A18c), whereas none of the A19 lines conferred expression that could be induced by the bacterial injection. Just like in larvae, some constitutive expression was observed in A19b and A19c, most likely due to the influence of tissue-specific enhancers at the site of integration of the transgene. We conclude that neither the *cecκB* site nor the R1 site is sufficient for promoting efficient *CecA1* expression from an otherwise intact upstream region. Our data also indicate that the R1 and *cecκB* sites do not confer different developmental or tissue-specific regulation of the *Cec* genes but rather act in concert to gain high levels of *CecA1* gene expression. Thus, the R1 site is a

FIG. 3. Schematic representations of the *CecA1-lacZ* fusion genes. The constructs contain 760 bp of upstream region and 62 bp of untranslated region (open box) of the *CecA1* gene fused to a simian virus 40 leader (filled box), providing a translational start site in frame with the *Escherichia coli lacZ* coding sequence (cross-hatched box). Numbers refer to positions relative to the transcriptional start site. Plasmids A18 and A19 carry mutations in the R1 and κ B sequences, respectively (for sequences, see Materials and Methods). The A15 plasmid has a 40-bp deletion (from –105 to –69) including the R1 and κ B sites.

FIG. 4. Both the R1 and the *ceckB* sites are required for full *CecA1-lacZ* expression in transgenic larvae and flies. (A to E) Transgenic larvae were not injected (–) or injected (+) with a mixture of *E. cloacae* and *M. luteus* and assayed for β -Gal activity in situ. The micrographs show dissected fat body (fb) and salivary glands (sg) from noninjected A10 larvae (WT promoter) (A), bacterium-injected A10 larvae with strong staining in the fat body (B), A15 larvae (R1 and *ceckB* deletion) (C), A18 larvae (R1 mutant) (D), and A19 larvae (*ceckB* mutant) (E). (F) Quantitative measurements of β -Gal activity in extracts of noninjected (gray bars) or bacterium-injected (black bars) transgenic flies. The β -Gal activity was measured in extracts from decapitated flies and calculated in relation to the protein concentration. Three independent strains of pA18 (a to c) and pA19 (a to c) were analyzed. The graph presents the results from one representative experiment in a series of four independent experiments.

novel sequence motif that is crucial for *CecA1* expression during the systemic response to infection.

To test whether the spacing and orientation of the *ceckB* and R1 sites relative to the axis of the DNA double helix is important for gene activation, we made reporter constructs with 5- and 10-bp spacer sequences inserted between the *ceckB* and R1 motifs. This should generate a half turn (5 bp) or a complete turn (10 bp) of the DNA helix between the sites. These constructs were tested in transient transfection assays, and they promoted transcriptional activation at the same level as the WT constructs (data not shown). This supports the conclusion that the *ceckB* and R1 motifs are distinct sequence motifs to which independent regulatory factors bind.

It has previously been shown that the *ceckB* motif is a target for the *Drosophila* Rel protein Relish (37). Since the R1 sequence shares considerable homology with the *ceckB* sequence (Fig. 1A), we speculated that distinct combinations of the Rel factors might interact with the *ceckB* and R1 sequences, possibly in response to infection by different classes of microorganisms. To investigate this, we prepared nuclear extracts from WT and mutant flies after infection. As shown by EMSA, nuclear extracts from WT flies contain both RBA and κ BA (Fig. 5, lanes 2 to 13), of which neither can be observed in unchallenged flies (Fig. 5, lanes 1), indicating that both the RBA and κ BA are induced in response to the infection. Interestingly, the kinetics of induction differ between the RBA and κ BA in response to infection by different microbes. The RBA peaks at 1 h after infection with the gram-negative bacterium *E. cloacae* and the gram-positive bacterium *M. luteus* (Fig. 5A, lanes 7 and 11), whereas the κ BA peaks at 3 h after infection

with the same bacterial species (Fig. 5B, lanes 8 and 12). In response to the yeast *S. cerevisiae*, both the RBA and κ BA were present in nuclei already at 15 min after infection (Fig. 5, lanes 2) and still persisted at 7 h after infection (Fig. 5, lanes 5). The different induction kinetics of the RBA and κ BA in response to bacteria support the conclusion that they consist of independent DNA-binding complexes. The fast induction kinetics of RBA may indicate that it plays a specific role in the early phase of *Cec* gene expression and that the R1 site is crucial for an immediate response to infection.

Cotransfection experiments in the *Drosophila* hemocytic cell line mbn-2 with the *Drosophila* Rel proteins Dorsal, Dif, and Relish confirmed previous reports demonstrating that all three Rel proteins can activate the WT *CecA1* promoter (Fig. 6A to C, A10) (7, 29). Mutations in either the R1 or *ceckB* motif reduced Relish-driven reporter gene expression to 10% of the activity on the WT promoter (Fig. 6A), demonstrating that both these elements have to be intact for Relish to activate *CecA1* expression. Dorsal and Dif *trans*-activations were also strongly affected by mutations in the R1 and *ceckB* sites (Fig. 6B and C). Mutations in the R1 site alone decreased Dif and Dorsal *trans*-activation to about 50% of that of the WT promoter. It should be noted that there are several additional κ B-like sites further upstream in the *CecA1* promoter region, which are present in all these constructs, with which different combinations of Rel factors may interact. Our conclusion is, however, that all three Rel factors require intact *ceckB* and R1 sites in the proximal promoter region for strong activation of the *CecA1* promoter, suggesting that these sites are targets for Rel protein interaction, either directly or indirectly.

FIG. 5. The RBA and κBA constitute independent DNA-binding activities that are induced with different kinetics in response to infection. EMSA with nuclear extracts from flies incubated with ^{32}P -labeled R1 probe (A) or cecκB probe (B). Flies were injected with a suspension of live bacteria or *S. cerevisiae* and incubated for the time indicated before making nuclear extracts.

To test for direct binding of the Rel proteins to the R1 and cecκB sequences, we made use of plasmids encoding tagged versions of the three *Drosophila* Rel proteins (12; Wickberg, unpublished). After transfection of these plasmids into mbn-2 cells, we analyzed the presence of RBA and κBA in total protein extracts. Cells transfected with FLAG-tagged Relish (FLAG-Relish) produced a strong, LPS-inducible κBA (Fig. 7A, lanes 1 and 2), which could be supershifted both with a FLAG antibody (Fig. 7A, lane 4) and with a Relish antibody

(data not shown), confirming the previously reported result that Relish binds to the cecκB motif in the *CecA1* gene (37). Extracts from cells transfected with plasmids expressing V5-tagged Dif (V5-Dif) or Dorsal (V5-Dorsal) revealed an LPS-inducible κBA (Fig. 7A, lanes 6 and 10), but since it could not be shifted with the V5 antibodies (Fig. 7A, lanes 8 and 12), it strongly indicated that neither Dif nor Dorsal is a component of the κBA under these conditions. To ascertain that the V5-Dif and V5-Dorsal were expressed in the mbn-2 cells, we used

FIG. 6. Both the R1 and the *ceckB* sites are necessary for full trans-activation of *CecA1* by Relish, Dif, and Dorsal. Relative β -Gal activity in *mbn-2* cells after cotransfection of WT and mutant *CecA1-lacZ* constructs and the expression plasmid pAct-Relish (A), pAct-Dif (B), or pAct-Dorsal (C) is shown. The cells were treated with (+) or without (–) LPS 4 h prior to harvest. The results shown are the mean values of the results from two independent experiments with standard deviations indicated by error bars.

Western blot analysis. This showed that high levels of the tagged proteins were present in the extracts used for supershift assays (data not shown). The nonshifted κ BA (Fig. 7A, lanes 6, 8, 10, and 12) is composed of endogenous Relish present in the *mbn-2* cells. The same transfected extracts contained a nuclear RBA, like that shown in Fig. 2A. The RBA did not increase in abundance after overexpression of the tagged Rel proteins, nor could it be shifted by the addition of the Flag or V5 antibodies

(data not shown), indicating that none of the Rel proteins are major components of the RBA.

Although our data show that the R1 and the *ceckB* sites are both required for Rel protein-mediated *CecA1-lacZ* expression (Fig. 6), we could only demonstrate direct interaction between Relish and the *ceckB* site (Fig. 7A). Neither V5-Dif nor V5-Dorsal interacted with the *ceckB* sequence, and none of the tagged Rel proteins bound the R1 motif. This suggests that Rel protein-mediated activation via the R1 and *ceckB* sites occurs indirectly or via protein-protein interactions. One may envisage that Relish/Dif and Relish/Dorsal heterodimers could bind the *ceckB* and/or R1 site with higher affinity than the respective homodimer. To test for this possibility, we carried out cotransfection experiments. EMSAs with cotransfected FLAG-Relish and either V5-Dif or V5-Dorsal revealed, as expected, an LPS-inducible κ BA (Fig. 7B, lanes 2 and 8), which was supershifted with the FLAG antibody (Fig. 7B, lanes 4 and 10). Addition of the V5 antibody did not produce any strong supershift, but a weak band could be observed (Fig. 7B, lane 6 and 12), indicating that some heterodimeric complexes between Relish/Dif and Relish/Dorsal also interact with the *ceckB* sequence in transfected cells. In contrast, the RBA present in the same extracts did not show any signs of changes in the presence of the FLAG or V5 antibodies (data not shown), again showing that the RBA is not composed of any of the Rel proteins.

One may argue that overexpression of tagged proteins in a cell line is a rather artificial situation that does not fully reflect the normal interactions in a cell. Therefore, we investigated whether the Rel proteins are components of RBA and κ BA during the normal course of an infection by analyzing nuclear extracts of mutant flies, which lack a functional copy of either of the *Relish* (*Rel*), *Dif*, and *Dorsal* (*dl*) genes. The κ BA was present in nuclear extracts of WT flies infected with gram-negative or gram-positive bacteria (Fig. 8A, lanes 2 and 3) and could be supershifted with Relish antibodies (Fig. 8A, lanes 7 and 8). However, in homozygous *Rel* mutant flies, the κ BA was absent (Fig. 8A, lanes 4 to 6). These data demonstrate that Relish is a component of the nuclear κ BA in flies. In contrast, the RBA present in nuclear extracts of infected flies (Fig. 8B, lanes 1 and 2) was not affected by the lack of a functional *Rel* gene (Fig. 8B, lane 5 and 6), strongly indicating that Relish is neither a component of the RBA nor is it required as an indirect activator of any RBA component. Extracts from homozygous *Dif* and *dl* mutant flies held both RBA (Fig. 8B, lanes 3, 4, 7, and 8) and κ BA (data not shown), showing that Dif and Dorsal are not components of the nuclear RBA or κ BA present in WT flies. In conclusion, our data strongly indicate that the R1 motif is a novel *cis*-acting element, which is required for expression of the *Cec* genes, and that the factors interacting with the R1 sequence are not members of the Rel family.

DISCUSSION

Insects, which have an open circulatory system, would be expected to be vulnerable to microbial infection, since their hemocoel is a perfect environment for a microorganism to grow and multiply. It is rich in nutrients and serves as a niche that is isolated from competing microbes. However, insects are not especially susceptible to microbial infections. In fact, we can, in an experimental situation, inject vast numbers of live

FIG. 7. Neither Relish, Dif, or Dorsal is a component of RBA in *mbn-2* nuclear extracts, whereas Relish, but not Dif or Dorsal, is a component of the κBA in the same extracts. (A) EMSA with nuclear extracts from *mbn-2* cells transfected with Relish-FLAG, Dif-V5, or Dorsal-V5 incubated with ³²P-labeled *ceckB* probe. (B) Cotransfections of Relish-FLAG-Dif-V5 and Relish-FLAG-Dorsal-V5. Mock refers to cells transfected with an empty pAct5C vector, showing that the FLAG (F) and V5 antibodies do not react with any endogenous protein in these cells. –, absent; +, present.

bacteria into a *Drosophila* larva or fly, and surprisingly enough, it will survive this massive attack. Part of the explanation for this resistance to microbial infection is the immediate expression of a large number of *Drosophila* immune-regulated genes (DIRG) (1, 5). One group of these DIRG codes for antimicrobial peptides. It has been known for a decade, that the κB sequence, originally identified in the immunoglobulin(κ) genes of mammals (26), is a crucial promoter element in many of the antimicrobial peptide genes in insects (10, 19, 38). However,

the DIRG are expressed with different kinetics in response to microbial challenge (2) and to different degrees, depending on the type of infecting agent (6, 18). Thus, it is likely that a number of different promoter elements are involved in the regulation in response to infection and that the number of binding sites and the relative affinity for these sites by different activating complexes dictate the specific pattern and kinetics of DIRG expression. Our data indicate that the R1 sequence is one such promoter element, which acts in parallel with the

FIG. 8. Nuclear κ BA is induced upon infection in WT flies, but absent in *Relish* mutant flies, while nuclear RBA is unchanged in the same extracts. WT (Or^R) and homozygous mutant flies (*Rel* mutant, *Dif* mutant, and *dl* mutant) were injected with a suspension of growing *E. cloacae* (G-) or *M. luteus* (G+) and incubated for 1 h. Nuclear extracts made from uninfected (-) or infected (G- or G+) flies were subjected to EMSA with 32 P-labeled ceckB probe (A) or R1 probe (B).

ceckB site to confer transcriptional activation in response to infection. As neither the ceckB site nor the R1 site was sufficient for promoting efficient *CecA1* expression from an otherwise intact upstream region, we conclude that factors interacting with the ceckB and R1 elements act jointly to gain high levels of *CecA1* gene expression.

The nuclear RBA was activated not only by gram-negative bacteria but also by yeast cells and gram-positive bacteria as well as by LPS and peptidoglycan fragments. Interestingly, the induction kinetics of the RBA and the κ BA differed depending on the infecting agent. In response to the yeast *S. cerevisiae*, nuclear RBA and κ BA appeared faster and persisted for a longer time than in response to bacteria. There was also a difference in the

TABLE 1. R1 sites in the 5' upstream region of inducible antimicrobial peptide genes in *D. melanogaster* and *D. virilis*

Gene	CG no.	Position	Sequence ^a
<i>D. melanogaster</i>			
<i>CecA1</i>	CG1365	-102	GTGTACTTT
<i>CecA2</i>	CG1367	-97	GTGTACTTT
<i>CecB</i>	CG1878	-93	GTGAACCTT
<i>CecC</i>	CG1373	-274	CTGTACTTg
<i>Def</i>	CG1385	-150	GTGAACCTa
		-778	GTGTACTTg
<i>Mtk</i>	CG8175	-872	CTGCACCTT
		-910	GaGCACTTT
<i>Drs</i>	CG10810	-48	GTGCACCTaT
		-580	CTGTACTTT
		-985	GTtTACTTT
<i>Drs</i>	CG10812	-751	GTtAACTTc
		-504	tTGCACCTT
<i>AttA</i>	CG10146	-86	GTtTACTTc
		-427	GTGAAGTTT
<i>AttC</i>	CG4740	-419	tTGAACCTa
<i>AttD</i>	CG7629	-323	GcGTACTTT
<i>D. virilis</i>			
<i>Cec1</i>		-118	GTGTACTTT
<i>Cec2A</i>		-119	GTGTACTTT
<i>Cec2B</i>		-126	CTGCACCTg
		-160	GTGTACTTT
<i>Cec3</i>		-227	CTtTACTTT

^a Uppercase letters indicate the consensus sequence of the R1 site, which is (G/C)TG(T/A/C)ACTTT.

peak of RBA and κ BA in response to bacteria. Both were present in the nuclei of infected flies 1 h after injection of bacteria, but at 3 h postinfection, the κ BA had increased further while the same extracts held very little RBA. This correlates with our conclusion that the RBA and κ BA are independent DNA-binding complexes. The difference in RBA and κ BA kinetics might reflect that different signal transduction pathways regulate these DNA-binding activities. Another possible explanation is based on the fact that Relish, which is a major component of the κ BA, is strongly upregulated in response to infection (5, 7), most likely by positive autoregulation of *Rel* gene expression. This may lead to a stepwise increase of Relish in immunocompetent cells and, in the sustained presence of bacteria, to a gradual enrichment of nuclear κ BA. If this model is correct, it also implies that the component(s) of the RBA are not upregulated at the transcriptional level, as the RBA peaked at 1 h postinfection and then declined. In fact, except *Relish*, very few genes for transcription factors have been found to be upregulated in response to infection (2, 5).

How common is the R1 motif in promoters of inducible immune genes in *Drosophila*? We scored 500 bp of upstream sequence of all known antimicrobial peptide genes present in the *Drosophila* genome database. We identified the R1 sequence in a number, but not all, of the antimicrobial peptide genes from *Drosophila* (Table 1). This differs from the occurrence of κ B-like motifs, which have been identified in all inducible antimicrobial peptide genes in *Drosophila* studied so far. Therefore, the R1 motif seems to confer a more specific function in the activation of the immune genes. Interestingly, there is a correlation between the presence of an R1 sequence in the promoter region of these genes (Table 1) and high levels of induction in response to infection, as reported from studies with DNA microarrays (5, 18). In the *Drosomycin* genes for ex-

ample, we found consensus R1 motifs in two of the seven homologs. These two *Drosomycin* genes (CG10810 and CG10812) are highly upregulated in response to infection while the others are only moderately induced or even repressed (5). Another observation is that the genes for *Diptericin* and *Drosocin*, which are activated more or less exclusively by the Imd/Relish pathway, do not contain any consensus R1 sequence. The presence of a linked R1/*cecrB* pair, located at a similar distance from the transcriptional start site in the *Drosophila Cec* and *Def* genes and in the *D. virilis Cec* genes suggest that these genes are regulated by similar factors binding to these promoter elements. As the Defensin and Cecropin peptides are active against gram-positive bacteria and the RBA was induced by gram-positive bacteria (Fig. 5A) and by peptidoglycan (data not shown), it is tempting to speculate that the R1 site may play an important role in the induction of these genes in response to gram-positive bacteria. Future experiments will be designed to identify the molecular nature of the RBA and its function for inducible expression of antimicrobial peptide genes.

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