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Drosophila immunity: a comparative analysis of the Rel proteins dorsal and Dif in the induction of the genes encoding diptericin and cecropin

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ABSTRACT

In *Drosophila*, bacterial challenge induces the rapid transcription of several genes encoding potent anti-bacterial peptides. The upstream sequences of the diptericin and cecropin A1 genes, which have been investigated in detail, contain two, respectively one sequence element homologous to the binding site of the mammalian nuclear factor κB. These elements have been shown to be mandatory for immune-induced transcription of both genes. Functional studies have shown that these κB-related elements can be the target for the *Drosophila* Rel proteins dorsal and Dif. Here we present a comparative analysis of the transactivating capacities of these proteins on reporter genes fused to either the diptericin or the cecropin κB-related motifs. We conclude from our results: (i) the κB motifs of the diptericin and cecropin genes are not functionally equivalent; (ii) the dorsal and Dif proteins have distinct DNA-binding characteristics; (iii) dorsal and Dif can heterodimerize *in vitro*; (vi) mutants containing no copies of *dorsal* and a single copy of *Dif* retain their full capacity to express the diptericin and cecropin genes in response to challenge.

INTRODUCTION

The powerful insect host defense involves the synthesis by the fat body and some blood cells of a battery of large-spectrum antimicrobial peptides (reviewed in 1,2). The synthesis of these molecules is induced within 30–60 min following septic injury and persists for one to several days. The peptides are secreted into the hemolymph where they act to kill invading microorganisms. Up to 100 antimicrobial peptides have been characterized from various insects sources and the genes encoding some 20 peptides have been cloned, predominantly in *Drosophila* (3). It came as a surprise, when the first upstream regions of these genes were sequenced, to observe that they contain numerous motifs which are homologous to *cis*-regulatory elements involved in the control of expression of genes of the mammalian acute phase response (4,5; reviewed in 1,3). Particularly striking among those motifs are decamers homologous to the binding site for the mammalian NF-κB (reviewed in 6). The functional relevance of these κB-related motifs in the insect host defense has been investigated in some detail in two model systems in *Drosophila*: (i) the induction of the gene encoding the 83 residue anti-Gram negative polypeptide diptericin (7); and (ii) the induction of the cecropin A1 gene (8). The diptericin promoter contains two identical κB-related motifs (hereafter referred to as κB-dipt) harboured within two 17 bp repeats (–43 to –60; –139 to –156; see ref. 5). Interestingly, these repeats are conserved, both in sequence and in their relative position in the diptericin promoter of several *Drosophila* species (3). The cecropin A1 upstream region contains only one κB-related motif GGGGATT*TT*T, (hereafter referred to as κB-cec) which differs from κB-dipt GGGGATT*CC*T; whereas κB-dipt has two canonical C nucleotides in the 3′ region, in κB-cec these nucleotides are replaced by Ts, which is exceptional for κB sites in mammals and insects (reviewed in 6). The sequences which are contiguous to κB-dipt and κB-cec in their respective promoters are also different and most noticeably, the proximal κB-dipt motif in the diptericin promoter partly overlaps sites homologous to IL-6 and interferon response elements (9), which is not the case for the κB-cec motif. Experiments based on transfection of an immune-responsive tumorous blood cell line from *Drosophila* (*mbn-2* cells; 10) have established that multimerized κB-dipt or κB-cec can confer LPS-inducibility to a reporter gene (7,11), in contrast to sequences mutated in the canonical three G residues in 5′ of the decamer. In a comprehensive functional study of the diptericin promoter through establishment of 60 transgenic fly lines, Meister and associates showed that replacement of the two κB motifs by random sequences, in an otherwise wild-type context, abolished the immune responsiveness (12). In these experiments, a single copy could mediate a severely reduced induction, indicating that cooperativity between the two motifs is essential for normal induction of this gene. Multimerized κB-dipt sequences within a minimal promoter context could confer immune-inducibility to a reporter gene in transgenic fly lines (12). In the case of the cecropin genes, experiments using the transgenic approach showed that the 760 bp region upstream of the site of transcription initiation, which contains the κB-cec motif, was able to confer immune-inducible expression of this gene; replacement or mutation of the κB-cec motif have not been reported under *in vivo*

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conditions but in transfection experiments, a reporter gene fused to this upstream region lost its LPS-inducibility when the κB-cec sequence was eliminated (8).

The structural and functional similarities between κB motifs in insect and mammalian immune-response gene promoters prompted the hypothesis that the *trans*-activating proteins binding to these motifs in insects could be related to mammalian NF-κB, which was first characterized in B lymphocytes as a nuclear protein that binds specifically to a 10 bp sequence (κB motif) in the κ light chain intronic enhancer (reviewed in ref. 13). NF-κB is classically described as a heterodimer of p50 and p65 subunits, which are members of a family of inducible transcription factors referred to as the Rel family. In *Drosophila*, the maternal effect gene *dorsal*, initially characterized as a key regulator of dorso-ventral patterning in early embryonic development (14, reviewed in 15), belongs to this family. Surprisingly, Reichhart and associates (16) showed that the dorsal gene was also expressed in larvae and adults of *Drosophila* and that immune challenge enhanced its expression. In addition, these authors reported that this challenge induced a nuclear translocation of the dorsal protein, a hallmark of all Rel family proteins which are normally sequestered in the cytoplasm by binding proteins containing ankyrin motifs (IκB family; cactus; reviewed in 6). Finally, in transfection studies with the immune-responsive tumorous blood cell line *mbn-2*, it was observed that the dorsal protein could sequence-specifically activate a reporter gene via the κB-dipt motif. Concomitant and independent studies by Ip and associates led to the characterization of a novel Rel protein in *Drosophila*, referred to as Dif for dorsal-related immune factor (17). The two proteins share 48% sequence homology in their N-terminal domains, the Rel homology domain, which is conserved among all members of the Rel family. They are totally distinct in their C-terminal domains. The Dif gene is not (or minimally) expressed in early embryogenesis. It is transcribed in fat body and blood cells and its expression is enhanced upon immune challenge. Like the dorsal protein, Dif is rapidly translocated into the nucleus after bacterial challenge. Finally, in co-transfection experiments of *mbn-2* cells, Dif expression vectors were able to induce a reporter gene *via* wild-type κB-cec sequences, whereas mutated motifs were inactive. In these experiments, dorsal was found to be a significantly less efficient *trans*-activator than Dif (18). Preliminary data in which dorsal and Dif expression vectors were compared for their efficiency to induce a reporter gene *via* κB-dipt motifs, in contrast, pointed to a stronger effect of dorsal (Georgel and Kappler, unpublished). To resolve this apparent contradiction, we have undertaken a series of experiments in which dorsal and Dif were compared on either κB-dipt and κB-cec motifs. We have also defined in more detail the promoter context in which κB-dipt is active. We have asked whether dorsal and Dif can heterodimerize and have investigated the inducibility of the immune response in mutants which supposedly affect the expression/function of Dif. Taken together, our results indicate that the two κB motifs, κB-dipt and κB-cec, have distinct functional characteristics. Our data also suggest that it is premature to consider either dorsal or Dif as a key activator of the antimicrobial genes in *Drosophila*.

MATERIALS AND METHODS

Plasmids

The pPAC-dorsal expression vector was described previously (16). pPAC-Dif was constructed by inserting a *Xho*I–*Not*I fragment from pSK-Dif (17) into the pPAC expression vector (19,20) digested with *Xho*I and *Not*I.

Reporter plasmids used in transfection assays were made in the pFLASH II vector (Synapsis) in which the firefly *Luciferase* gene is driven by the HSV *Thimidine kinase* (tk) gene promoter. Plasmids 8 κB-Luc, 8 PRI-Luc, 8 PRI mut κB-Luc and 8 PRI mut GAAANN-Luc were described in ref. 21. For the plasmid referred to as 8 cec-Luc, eight copies of the oligonucleotide 5′-ATCGGGGATTTTTGCAGAGAAAA-3′ were cloned head-to-tail between the *Bam*HI–*Bgl*II sites of the vector.

For the GST–Dif expression vector, the 1200 bp *Nde*I–*Eco*RV fragment (encoding amino acids 17–526 of Dif) was filled in with Klenow enzyme and subcloned into the *Sma*I site of pGEX3T (Pharmacia). The GST-dorsal vector was constructed by inserting a 1200 bp *Eco*RV–*Sac*I fragment (encoding amino acids 2–402 of dorsal) from a GST-dorsal-containing pPAC vector into pGSTag (Pharmacia) digested with *Eco*RI (and filled in) and *Sac*I.

Cell cultures, transfection experiments and luciferase activity quantification

Tumorous blood cells (*mbn-2*; ref. 10) were grown to 80% confluent monolayers at 25°C in Schneider's medium (Sigma) supplemented with 10% fetal calf serum (Gibco-BRL), 10^5 U/l penicillin and 100 mg/l streptomycin.

Cells were transfected by the transfection reagent DOTAP (Boehringer, Mannheim) using 1 or 2 μg of reporter plasmid (see figure legends) and 1 μg of the β-galactosidase expression vector pACH110 (19) as internal control for the transfection efficiencies. The amounts of the co-transfected expression vectors are indicated in the figure legends. After 6 h, the cells of each dish were washed and incubated for 48 h. Cells were lysed during 20 min in the reporter lysis buffer (Promega) and luciferase activity was measured in a luminometer (BCL Book, Promega) immediately after addition of the substrate (Luciferin, Promega) according to the protocol of the distributer. β-galactosidase activity in the cell lysates was measured using *O*-nitro-phenol-β-D-galactoside as substrate and the values were used to normalize variability in the efficiency of transfection.

Production of recombinant proteins

The GST–dorsal fusion protein was expressed in LE 392 and purified using a batch procedure exactly as described in ref. 22. For the expression and purification of the GST–Dif protein, we used standard procedures. A 100 ml culture of bacteria (BL 21) was grown to an OD of 0.6 at 37°C. After induction with IPTG (0.3 mM) and 6 h culture at 30°C, cells were pelleted by centrifugation, washed with cold PBS and resuspended in 5 ml lysis buffer (PBS, 0.1 mM PMSF, 1% Triton, protease inhibitors). Bacteria were sonicated (30 s; seven times) and centrifuged at 12 000 *g* for 10 min at 4°C. The supernatant was loaded on a 1 ml glutathione–Sepharose 4B (Pharmacia) column, washed with 10 vol PBS–Triton 1%, 10 vol 50 mM Tris–HCl pH 8 and fusion proteins were eluted with 10 ml of elution buffer (50 mM Tris–HCl pH 8, 10 mM glutathione). Fractions of 1 ml were collected and proteins were quantified with a Bradford colorimetric assay (Bio-Rad).

The pAR-dl recombinant protein was expressed in BL 21. Bacteria were grown to an OD of 0.5 and induced with 2 mM IPTG. After a 3 h culture at 37°C, cells were collected by a 5 min centrifugation at 5000 *g* and resuspended in 1/10 vol extraction buffer (25 mM HEPES pH 7.9, 100 mM KCl, 12.5 mM $MgCl_2$,

1 mM DTT, 1 mM PMSF, 0.1% NP-40, 20% glycerol, 0.1 mg/ml lysozyme, protease inhibitors). Cells were incubated for 30 min at 4°C followed by 5 min at 37°C, subjected to three freeze–thaw cycles and sonicated (two times, 1 min). After a 30 min centrifugation at 12 000 *g* on the same volume of a sucrose solution (40% sucrose, 10 mM Tris–HCl pH 7.5, 1 mM EDTA, 200 mM NaCl) the pellet containing inclusion bodies was dissolved for 30 min at 4°C in 1/100 vol extraction buffer supplemented with 4 M guanidine–HCl. Proteins were dialysed for 3 h against extraction buffer containing 3 M guanidine–HCl and for 3 additional hours against extraction buffer. Insoluble proteins were removed by a 10 min centrifugation at 10 000 *g* and the supernatant was aliquoted and stored at 4°C.

Electrophoretic mobility shift assays

Oligonucleotides, which were end-labelled using [γ-^{32}P]ATP and T4 kinase, were gel-purified.

The gel shift assays were performed as described in ref. 23. Purified GST fusion proteins (300 ng) were diluted in 20 µl binding buffer (10 mM HEPES pH 7.9, 50 mM NaCl, 1 mg/ml bovine serum albumin, 3 mM $MgCl_2$, 8 µg/ml poly(dI·dC)–poly(dI·dC), 6 mM β-mercaptoethanol, 10 mM EDTA, 10% glycerol). After addition of 100 000 c.p.m. labelled probe (10 fmol), the reaction was incubated for 10 min at room temperature. The mixture was then loaded onto a 4% polyacrylamide gel in TGE buffer (25 mM Tris base, 190 mM glycine, 1 mM EDTA). These conditions are crucial to detect the binding of the GST-dl protein on the Zen probe, but we have observed that the GST–Dif fusion protein does not require so strict binding conditions: we can detect a comparable binding on the various probes in our classical gel-shift conditions (see ref. 7). Control experiments were performed with the GST protein alone with which no retarded complex was observed with any of the probes (data not shown). For competition experiments, increasing molar excess (10-, 50- and 100-fold excess) were added to the mixture immediately after the addition of the labelled probe. Quantification of the binding was done using a phosphorimager (Bio-imaging analyser BAS 2000; Fuji).

When performed with pre-purified *mbn-2* cells extracts (as it is the case in Figs 3B and 4), gel shifts were done exactly as described in ref. 7.

Immunoprecipitation and Western blot

GST-Dif fusion proteins (3 µg) were mixed for 3 h at 4°C with 50 µl glutathione–Sepharose 4B resin equilibrated in 300 µl interaction buffer (20 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, 1 mM DTT, 0.5 mM PMSF). Protein extracts (5 µg) from pAR or pAR-dl expressing bacteria were added and after 3 h incubation, the resin was collected by a 3 min centrifugation at 300 *g* and extensively washed three times with 500 µl interaction buffer. Beads were resuspended in 25 µl Laemmli buffer and precipitated proteins were analysed by 7.5% denaturing SDS–PAGE. After migration, proteins were electroblotted onto a nitrocellulose filter which was incubated for 1 h in blocking solution (5% low fat dry milk). The blot was probed with a monoclonal anti-dorsal antibody (used at a 1:20 dilution in TBS–Tween 0.1%) overnight at 4°C. The second antibody was a donkey anti-mouse horseradish peroxidase conjugate (Amersham Life Science) used at a 1:1000 dilution and the detection of the dorsal protein was done using enhanced chemiluminescence performed as recommended by the manufacturer (Amersham Life Science).

Drosophila stocks and culture

Oregon R flies were used as a standard wild-type strain. The *dorsal* mutant strains Def (2L) TW 119; dl^{D7} and In (2L) dl^{H}, which were obtained from the Tübingen stock center (24), were balanced with CyO. Stocks and crosses were maintained on standard corn meal medium at 25°C.

RNA preparation and analysis

For bacterial challenge, adult flies of the appropriate genotype were selected and pricked with a tungsten needle previously dipped into a concentrated culture of *Escherichia coli* and *Micrococcus luteus.* After 3 h, flies were collected and total RNA extracted using the TRIZOL^{R} method (Gibco-BRL). RNA samples were fractionated on denaturating 1% agarose–formaldehyde gels and transferred to nylon membranes (positive membrane, Appligene). The filters were sequentially hybridized with random-primed (rediprime, Amersham) labelled probes (diptericin and rp 49) or with a ^{32}P kinased oligonucleotide in the case of cecropin as described in ref. 25. Quantification was done using a phosphorimager system.

RESULTS

Transfection of a dorsal expression vector can substitute for LPS induction of a diptericin reporter gene in a tumorous blood cell line

The analysis of the proximal upstream region of the diptericin gene had revealed the presence of two 17 bp repeats distant by 96 nucleotides, which harbour a κB-related sequence (7). A DNAse I protection study, in which a 300 bp fragment of upstream sequence of the diptericin gene had been incubated in the presence of protein extracts from bacteria-challenged and control *Drosophila*, had shown that both 17 bp repeats were indeed protected by proteins from induced insects, but not from controls (9). The protection of the most proximal 17 bp repeat extended in fact over 30 nucleotides (–31 to –62) and covered, in addition to the κB-related sequence, a motif homologous to the mammalian NF-IL 6 response element and a GAAANN motif (26,27). The latter is present in the interferon sensitive response element of many interferon-stimulated genes in mammals (28,29). For simplicity, we will refer to this 30 nucleotide region hereafter as protected region I (PR I) and to the 17 bp repeat simply as κB-dipt although it contains 7 nucleotides in addition to the strict κB-dipt decamer.

Drosophila tumorous blood cells (*mbn-2* line; ref. 10) can be induced to express the diptericin and the cecropin genes by addition of lipopolysaccharide (LPS) to the culture medium (7,11). In a recent study we demonstrated that the high level of LPS-induced expression of a diptericin–luciferase reporter gene in these cells results from the cooperativity of the κB-related motif and the GAAANN sequence (21). We have asked here whether in this system the Rel protein dorsal can substitute for LPS-stimulation in inducing the transcription of the reporter diptericin–luciferase. We were also interested to know whether the possible effect of the dorsal protein was dependent on the cooperativity of the κB-related site with the GAAANN motif, as observed in the above-mentioned LPS-stimulation experiments (21). For this we have used four types of constructs in which the luciferase reporter gene was fused to either of: (i) multimerized wild-type κB-dipt sequences; (ii) multimerized

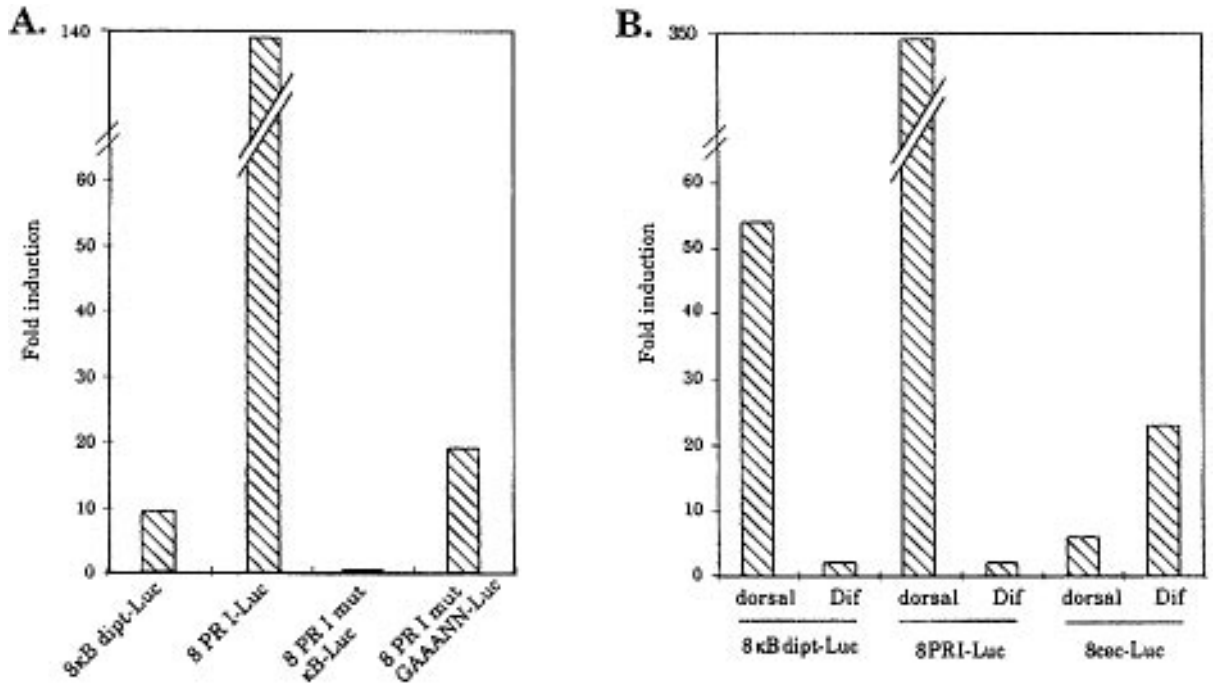


Figure 1. (**A**) The presence of the GAAANN motif in the Protected Region I is necessary for a high level of dorsal-mediated induction of a reporter gene.*mbn-2* cells were transiently transfected with 2 µg of the indicated Luciferase (Luc) reporter vectors and 2 µg of the dorsal expression vector (pPAC-dl). Fold induction represents the ratio between the luciferase activity measured in cells transfected with the pPAC-dl expression vector and the activity in cells transfected with the empty pPAC vector. (**B**) Dif and dorsal proteins manifest different *trans*-activation properties according to the κB-like target sequence. *mbn-2* cells were transfected with 1 µg of the indicated luciferase reporter plasmids, 2 µg of pPAC-dorsal or pPAC-Dif expression vectors. Note that the maximum value for the induction is different in the two panels; this reflects a different responsiveness of the cells between various experiments probably due to the heterogeneity of the*mbn-2* cell population. The values indicated in the figure were obtained in a representative experiment and each transfection was repeated three times.

wild-type PR I sequences which contain the three overlapping sequence motifs as explained above; (iii) multimerized PR I sequences in which the κB-related motifs had been mutated (GGG→ATT); (iv) multimerized PR I sequences carrying a mutation in the GAAANN (AAA→GTC) motif. The results are presented in Figure 1A. They confirm that in these conditions dorsal can transactivate the reporter gene via a κB-related motif as already pointed out by Reichhart *et al.* (16). Interestingly, the results demonstrate for the first time that the level of induction is considerably higher (>10-fold) with PR I promoter sequences than with κB-dipt. Mutating the κB-related motif in the PR I construct fully abolished the inducibility, whereas mutating the GAAANN motif noticeably reduced the level of dorsal-induced expression (6-fold) which remained nevertheless relatively high.

In essence, the data obtained with co-transfection of dorsal and the reporter constructs are similar to those obtained when LPS was used to stimulate the expression of the reporter gene.

A comparative analysis of the transactivation of κB-dipt and κB-cec by dorsal and Dif

We have next compared the effects of transfecting dorsal or Dif expression vectors on the diptericin promoter constructs carrying multimerized κB-dipt and PR I sequences. As illustrated in Figure 1B, Dif appeared as a less efficient transactivator than dorsal in these conditions. As stated in the Introduction, the upstream region of the cecropin A1 gene contains a κB-related sequence (which we refer to as κB-cec) which differs from κB-dipt by the replacement of two crucial C nucleotides by Ts in the 3′ region of the decamer (see Fig. 2A). We were therefore interested to extend the above experiments to the cecropin κB-related motif. For this, we have used a reporter plasmid containing a multimerized 24mer, corresponding to the wild-type sequence –75 to –95 of the cecropin A1 promoter and harbouring the corresponding κB-related motif. As illustrated in Figure 1B, both dorsal and Dif could transactivate this construct. However, in sharp contrast to the situation observed with κB-dipt, Dif proved to be more efficient than dorsal on κB-cec (4- to 5-fold). It is noteworthy that in these experiments, the level of induction conferred by Dif on κB-cec was lower than that of dorsal on κB-dipt.

Dif and dorsal do not produce similar gel-shifts with oligonucleotides containing κB-related sequences

We have next prepared recombinant Dif and dorsal proteins which were essentially truncated to their corresponding Rel domains (as GST fusion proteins, see Materials and Methods). Indeed, this domain has been shown in all Rel proteins to be responsible for DNA-binding and dimerization (6). The recombinant proteins were incubated with the following radiolabelled oligonucleotides (see Fig. 2A and above): (i) a single motif of κB-dipt; (ii) two copies of κB-dipt; (iii) the PR I sequence; (iv) κB-cec; (v) the κB-related motif of the zerknüllt (zen) gene promoter which reportedly is a strong binding site for dorsal (23). As illustrated in Figure 2B, Dif produced a retarded complex with all five oligonucleotides, the strongest signals being observed with two copies of κB-dipt (2× κB-dipt) and κB-cec. This result is in keeping with the competition experiment shown in Figure 2 (panel C, i) in

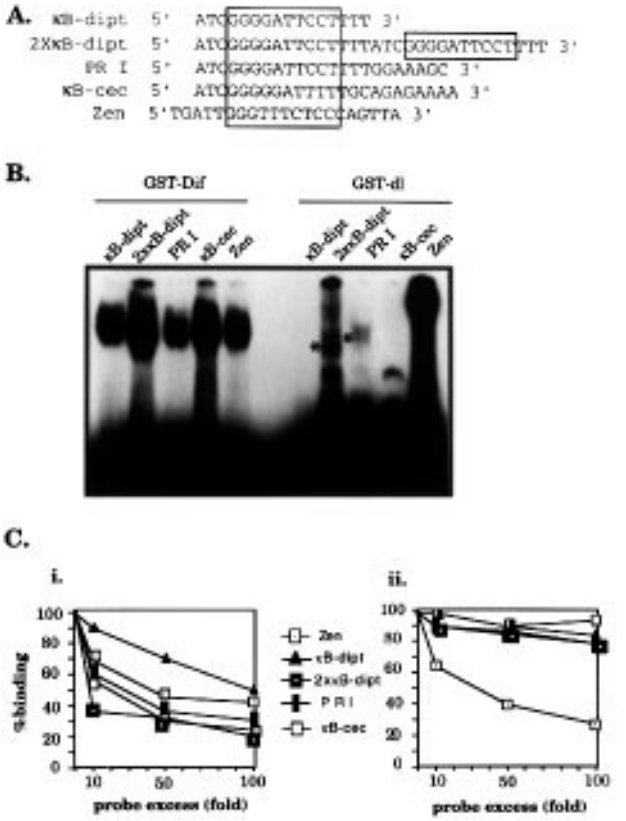


Figure 2. The GST–Dif and GST–dorsal proteins bind κB-like-containing probes with different affinities. (**A**) Sequences of the oligonucleotides used in gel shift experiments. κB-dipt is the motif which is found upstream of the diptericin gene and 2× κB-dipt is a dimer of this motif. PR I is an oligonucleotide corresponding to the Protected Region I of the diptericin promoter (see text), κB-cec is an oligonucleotide containing the κB-like motif which is present in the promoter of the cecropin A1 gene and Zen contains a strong dorsal binding site from the *zerknüllt* promoter. Only the upper strand is shown and the κB-like motif is boxed. (**B**) 300 ng of the recombinant GST–Dif or GST-dl proteins were incubated with 100 000 c.p.m. of the labelled probes and the retarded complexes were resolved in a gel shift assay. The specificity of the complexes marked with a star (*) was checked by competition experiments (data not shown). (**C**) The binding of the GST–Dif (i) or GST-dl (ii) on the labelled Zen probe was competed with an excess (10-, 50- and 100-fold excess) of each of the five cold probes as indicated in the figure. After quantification of the retarded bands, the results were plotted in graphics i and ii.

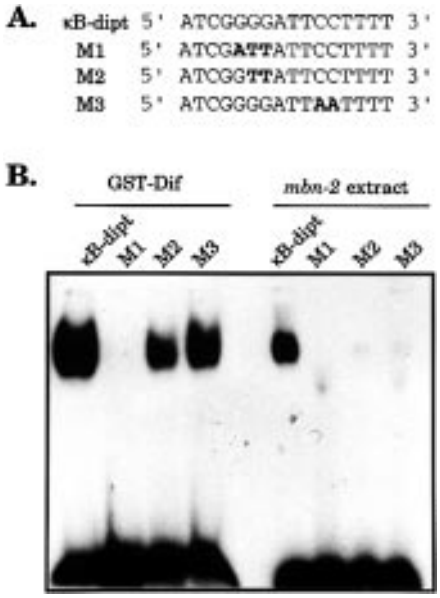


Figure 3. The recombinant GST–Dif protein and the LPS-inducible activity binding to the diptericin κB-like motif have different affinities for various mutated κB-like probes. (**A**) Sequence of the wild-type (κB-dipt) and mutated (M1, M2 and M3) oligonucleotides containing the κB-like motif of the diptericin promoter. The mutations are in bold-type letters. (**B**) Electrophoretic mobility shift assay using 300 ng of GST–Dif or 1 μg of a pre-purified LPS-induced *mbn-2* cells extract incubated with 20 000 c.p.m. of the indicated labelled probes.

which the various probes were separately added in excess under cold form to compete for the binding of Dif to the labelled Zen probe: all probes were able to compete this binding, the oligonucleotides 2× κB-dipt and κB-cec being the most efficient competitors.

These results differed markedly from those obtained with dorsal. In the latter case, a strong gel-shift signal was only observed with the Zen probe; the κB-dipt or κB-cec probes yielded no detectable gel shifts. Only the oligonucleotides containing two copies of the κB-dipt or the full PR I sequence induced a gel shift, albeit with a low signal intensity.

In conclusion, these results indicate that Dif can bind to any of the κB-related motifs (Zen, κB-dipt, κB-cec); a single motif is sufficient for binding and double copies (2× κB-dipt) yield a stronger signal. In contrast, dorsal, which binds strongly to the motif present in the zen promoter, does not detectably bind to the κB-related motif of the cecropin or the diptericin promoter. It can bind only to κB-dipt if this motif is duplicated, as is the case in the native diptericin promoter, or if κB-dipt is present in the special context of the PR I sequence of this promoter. Even under these circumstances, the binding is not as marked as for Dif (with the exception of the zen motif), to judge from the intensity of the signals presented in Figure 2B.

Dif forms with κB-dipt a complex different from that formed by protein extracts of stimulated blood cells

In the foregoing experiment, Dif gave a marked signal in gel shift assays with κB-dipt. As the nucleotide sequence of κB-dipt is different in two crucial positions from that of κB-cec (see above), we were interested to see which nucleotides were of paramount importance for binding of Dif to a κB-related motif. For this we synthesized three oligonucleotides corresponding to the 17 bp repeat of the diptericin promoter which harbours the κB-related motif. Within this motif we introduced the following mutations (see Fig. 3A): (i) in 5′, mutation GGG to ATT (M1) or (ii) GG to TT (M2); (iii) in 3′, mutation CC to AA (M3). As shown in Figure 3B, mutating the 5′ three G nucleotides abolished binding of Dif to the mutated κB-dipt. Mutation M2 decreased the gel shift signal, which however remained conspicuous (25% of binding remains). Mutating the 3′ CC residues only moderately (40%) affected the intensity of the gel shift signal. These results demonstrate the importance of the 5′ G residues for the binding of Dif to the κB-related motif and conversely they show that the two C nucleotides in 3′ can be replaced by A residues without a deleterious effect on binding.

It has been proposed that the LPS-induced DNA–protein complex formed with κB-related motifs and *mbn-2* cell extracts

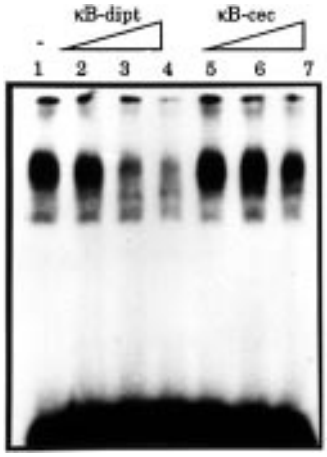


Figure 4. The binding capacities of κB-dipt and κB-cec are not interchangeable. One microgram of a pre-purified nuclear extract of LPS-induced *mbn-2* cells were mixed with 20 000 c.p.m. of the labelled κB-dipt probe without (–) or with increasing (10-, 50- and 100-fold excess) amounts of cold κB-dipt or κB-cec oligonucleotides. Complexes were resolved in a gel shift assay.

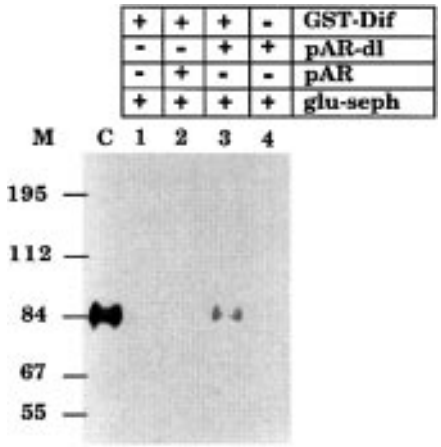


Figure 5. Direct protein–protein interaction between the recombinant GST–Dif and Dorsal. Purified GST–Dif proteins were first attached to glutathione–Sepharose beads (glu-seph) and then incubated with bacterial extracts from pAR or pAR-dl transfected cells. After centrifugation, the protein content of the pellet was analysed by Western blot revealed with a monoclonal anti-dorsal antibody. C, control lane containing 3 μg of dorsal protein; M, pre-stained molecular weight markers (in kDa).

reflects the binding of Dif or at least involves the presence of Dif (30). To test this hypothesis, we have incubated under the same conditions LPS-stimulated *mbn-2* extracts with wild-type κB-dipt and mutated oligonucleotides M1, M2 and M3 (Fig. 3B). The protein extracts clearly formed a gel shift with the wild-type κB-related motif. The motif mutated in the three G residues (M1) gave no signal as observed when Dif was incubated with this probe. In contrast, however, to the above experiments with Dif, cell extracts incubated with probe M2 (two Gs out of three mutated) and especially probe M3, in which the 3′ CC residues had been mutated, gave no signal (or an extremely faint signal which was determined to be 4% compared to the signal obtained with the wild-type probe). This result indicates that the nuclear activity binding to the κB-dipt in LPS-induced *mbn-2* cells differs from that of recombinant Dif since both exhibit a different behaviour with respect to the mutated probes.

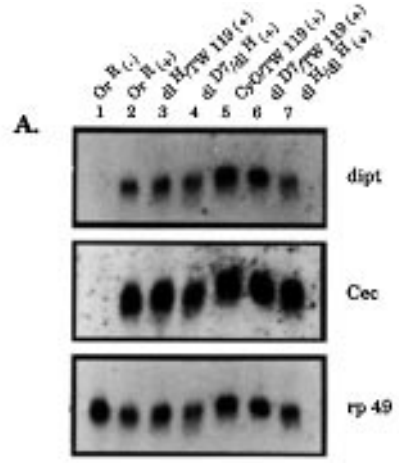
Finally, we have compared the capacity of κB-dipt and κB-cec to compete for the nuclear binding activity formed when labelled κB-dipt was incubated with protein extracts from induced *mbn-2* cells. As illustrated in Figure 4, an excess of homologous unlabelled probe efficiently competed the binding activity whereas, in marked contrast, excess of unlabelled κB-cec was unable to compete this binding. This result indicates that the composition of the complex bound to the diptericin κB-related element differs from that which binds to the κB-cec motif.

Dif and dorsal recombinant proteins can heterodimerize *in vitro*

Given that mammalian Rel proteins have been repeatedly demonstrated to be able to form heterodimers (6), we investigated whether dorsal and Dif could heterodimerize. For this we took advantage of the presence of the GST moiety in our Dif fusion protein to specifically precipitate the recombinant protein with glutathione-coupled Sepharose beads. In a pilot experiment, bacterial dorsal protein [which contains no GST sequence since it was produced with the pAR-dl expression vector (23,31)], was added to a mixture containing GST–Dif proteins and glutathione–Sepharose resin. After incubation and centrifugation, the protein content of the pellet was analysed by SDS–PAGE. The blot was incubated with a monoclonal anti-dorsal antibody (gift from Prof. R. Steward) and was revealed with enhanced chemoluminescence. As seen in Figure 5, dorsal protein was only detected by the antibody when it was incubated in the presence of GST–Dif (lane 3), and not in the other conditions which served as controls.

The expression of the diptericin and cecropin genes is not markedly affected in several combinations of dorsal mutants

It was previously shown that the diptericin and cecropin gene expression is not affected in dorsal-deficient mutants (25). Several explanations were forwarded for this result. In particular it was proposed that Dif could be the paramount *trans*-activator regulating antibacterial gene expression via kB-related motifs (30). To date, no Dif-deficient mutants have been described. However, given that the dorsal and Dif proteins can heterodimerize *in vitro* as shown above, we were interested to study the expression of the immune-inducible genes in several heterozygous combinations which supposedly affect the titre of Dif. For this we used three types of strains (see Materials and Methods for a full description of the genotypes): (i) flies carrying a deficiency (TW 119) uncovering both the dorsal and Dif genes (R. Steward, personal communication); (ii) dl^{H} flies in which the dorsal gene was disrupted by an inversion; and (iii) dl^{D7} flies in which a point mutation in the Rel domain of *dorsal* results in the replacement of Arg 63 by a Cys residue in the protein (32). The modified protein is able to inactivate a wild-type copy of dorsal by forming heterodimers which are not able to bind DNA. As a consequence the mutant has a dominant-negative effect, leading to weakly dorsalized embryos (33). The three types of mutants were crossed in various combinations. The offspring was submitted to bacterial challenge and the expression of the diptericin and cecropin genes was monitored by Northern blot analysis; quantification was done using a phophorimager. The results are illustrated in Figure 6A and B. They confirm



B.

	Or^R (-)	Or^R (+)	dlH (+) TW 119	dlD7 (+) dlH	CyO (+) TW 119	dlD7 (+) TW 119	dlH (+) dlH
% dipt	9,8	100	88,1	105,4	137,9	135,3	83,9
% cec	6,9	100	94,0	76,7	73,8	94,1	91,0
Status of dl and *Dif* genes	2 *dl* 2 *Dif*	2 *dl* 2 *Dif*	0 *dl* 1 *Dif*	1 *dl mut* 2 *Dif*	1 *dl* 1 *Dif*	1 *dl mut* 1 *Dif*	0 *dl* 2 *Dif*

Figure 6. Diptericin and cecropin gene expression in various dorsal-mutant contexts. (**A**) Total RNA (10 µg) from control (–) or bacteria-challenged adults (+) was separated by gel electrophoresis, blotted to nylon membrane and hybridized successively with a diptericin cDNA probe (dipt), an oligonucleotide complementary to cecropins A1 and A2 (cec) and to a rp 49 cDNA probe (rp 49). (**B**) Signals were quantified with a phosphorimager system and the value (given in percentage) for diptericin and cecropins gene expression in each lane was normalized with the value of the rp 49 signal in the same lane. 100% was taken for the value obtained in the immunized wild-type Or^R flies (Or^R +).

first of all that the absence of any functional allele of *dorsal* (dl^H/TW 119, lane 3; dl^{D7}/dl H, lane 4; dl^H/dl^H, lane 7) does not significantly reduce the inducibility of the diptericin and cecropin genes by immune challenge. The results also show that in TW 119 flies balanced with CyO (lane 5) which contain one copy of *dorsal* and one copy of *Dif*, the expression of both diptericin and cecropin is not noticeably affected. As the expression of antibacterial genes is not affected in the absence of dorsal (see above), this result is compatible with the idea that a single copy of the Dif gene might be sufficient to induce the expression of the diptericin and cecropin genes and would imply that *Dif* is not haplo-insufficient. Finally, in the *trans*-heterozygous combination dl ^{D7}/TW 119 (lane 6), which contains one mutated allele of *dorsal* and one wild-type copy of *Dif*, the inducibility of the diptericin and cecropin genes remains also unaffected. The latter result suggests several possibilities: (i) dorsal and Dif do not heterodimerize *in vivo*, and thus, a single copy of Dif is sufficient to mediate an immune response; (ii) if the two Rel proteins can heterodimerize, it is possible that the dorsal–Dif complex is still able to bind DNA and if this is not the case (iii) one might consider that neither dorsal, nor Dif is strictly required for the inducible expression of the diptericin and cecropin genes.

DISCUSSION

The results presented in this paper confirm that the two Rel proteins dorsal and Dif can activate the transcription of reporter genes in tumorous blood cells via either the κB-dipt or the κB-cec motifs. The efficiency of transactivation was highest, in our conditions, when dorsal was co-transfected with κB-dipt-luciferase and Dif with κB-cec-luciferase. Dorsal indeed appears as a comparatively poor transactivator on a κB-cec motif and Dif on a κB-dipt motif. These results are in agreement with, and extend, previous studies from this laboratory and that of Engström and associates (18). We note with interest that the level of activation by dorsal in co-transfection experiments is significantly enhanced when it is allowed to act on a promoter element containing the GAAANN motif in addition to κB-dipt, which indicates that this Rel protein can engage in a process of cooperativity with another *trans*-activator, which in the present case, is most likely the 45 kDa GAAANN binding protein (21). Similar studies have not yet been performed with the cecropin promoter which also contains a GAAANN motif in close vicinity (5 nucleotides) to the κB-related sequence.

The data obtained in this study with gel shift experiments provide two essential and unexpected insights into this system. First, they show that dorsal and Dif differ in their requirements as regards the nucleotide sequence of the κB-related motifs. Indeed, recombinant dorsal produces a detectable gel shift only in the presence of a duplicate κB motif (or a multimerized construct), or in the special context of the PR I sequence. Single copies of κB-dipt or κB-cec do not allow binding of dorsal. In contrast, single copies are sufficient for binding of Dif. Moreover, the structural requirements for Dif binding to a single κB-related site are not very stringent as Dif gave a marked signal not only with wild-type κB-dipt motif, but also when the two 3′ CC nucleotides, or two out of the three 5′ G nucleotides, had been mutated. Taken together, these data point to markedly different binding characteristics for dorsal and Dif. We also note an apparent contradiction between these results and those obtained by transfecting Dif or dorsal expression vectors. Therefore, we propose that the transactivation properties of Dif, (which appears as a 'powerful binder and poor activator') or dorsal (a 'strong activator but poor binder') could be modulated by the association of these proteins with different partners. The second insight comes from the experiments with cell extracts which clearly show that the complexes formed with κB-dipt and κB-cec motifs have different protein compositions. This is in particular illustrated by the fact that the DNA–protein complex formed by κB-dipt and protein extracts from induced cells is competed by excess of homologous κB-dipt sequences but not of κB-cec.

Dorsal and Dif can heterodimerize, as shown by our *in vitro* experiments. Although it had been suggested that these two Rel proteins might heterodimerize, in view of the data obtained about heterodimerization of various mammalian Rel proteins, it had not been demonstrated up to now. Assuming that this might be the case under *in vivo* conditions, we generated mutants expressing a single copy of Dif and a single copy of a dominant-negative mutant dorsal protein. In these mutants, the inducibility of the diptericin and cecropin genes was not affected. In mutants containing a single copy of Dif, the level of induction of these antibacterial genes by immune challenge was similar to that of wild-type flies. This latter result indicates that, in the hypothesis that Dif functions as a transactivator for the antibacterial genes, the Dif gene is not haplo-insufficient.

What then have we learned in this and the preceding studies on the role of the Rel proteins dorsal and Dif in the immune response of *Drosophila*? Firstly, the genes encoding both Rel proteins are expressed in immuno-responsive tissues (fat body, some blood cells) of larvae and adults. Secondly, both proteins are translocated into the nuclei after bacterial challenge. And thirdly, both proteins can transactivate in co-transfection experiments in tumorous blood

cells a reporter gene via κB-related motifs. We have now shown that, in the case of the diptericin gene and the cecropin gene, the difference in nucleotide sequence between the respective κB sites is functionally relevant. Also that the two Rel proteins are not interchangeable, Dif being less stringent than dorsal in its structural requirements for binding to κB-related sequences. We have also demonstrated that, at least under *in vitro* conditions, both proteins can heterodimerize. From the early experiments with dorsal mutants, we had learned that the immune-induced expression of the antibacterial genes can take place in the absence of dorsal. In this study, we see that flies expressing no dorsal and a single copy of Dif still exhibit a normal inducibility of the antibacterial genes. Finally, we also see that the complex formed between proteins from induced blood cells and a κB-related motif has different binding characteristics than that formed by recombinant Dif and the same κB motif. These data raise the question whether the role of dorsal and/or Dif in the immune response is precisely the control of the expression of the antibacterial genes. It is our feeling that although most of the results reported over the last years are compatible with this much-heralded hypothesis, they do not conclusively prove its validity. It is our hope that a detailed genetic dissection of the signalling pathways and a biochemical study of the *trans*-activating proteins present in induced cells/tissues will eventually lead to a more refined picture in this field.

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