The *dorsal*-related immunity factor, Dif, is a sequence-specific *trans*-activator of *Drosophila* *Cecropin* gene expression

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A new member of the Rel family of transcription factors, the *dorsal*-related immunity factor, Dif, was recently cloned and suggested to be involved in regulating the immune response in *Drosophila*. Despite its classification as a Rel family member, the Dif cDNA-encoded product has not been proven previously to be a transcription factor. We now present evidence that the Dif gene product *trans*-activates the *Drosophila* *Cecropin A1* gene in co-transfection assays. The *trans*-activation requires a 40 bp upstream element including an insect κ B-like motif. A dimer of the κ B-like motif 5'-GGGGATTTT inserted into a minimal promoter conferred high levels of reporter gene expression by Dif, while a multimer of several mutated versions of this motif was not activated, demonstrating the sequence specificity of Dif. Full *trans*-activation by Dif requires the C-terminal part of the protein. The morphogen dorsal (dl) can also activate the *Cecropin A1* promoter, but to a lesser extent and in a less sequence-specific manner than Dif. Simultaneous overexpression of Dif and dl in co-transfection assays revealed that dl possesses a dominant negative effect on Dif *trans*-activation. This study establishes that Dif is a sequence-specific transcription factor and is probably a key activator of the immune response in *Drosophila*.

Key words: cecropin/*Drosophila*/invertebrate immunity/NF- κ B/Rel protein

Introduction

Insects defend themselves effectively against intruders such as bacteria, parasites and other microorganisms. Their immune system is in many ways reminiscent of the innate immune reactions in vertebrates, and it is possible that the two systems are related (Faye and Hultmark, 1993; Hultmark, 1993; Ip and Levine, 1994). The humoral insect immune system is inducible by bacterial infection and wounding, leading to a vast production of a number of bactericidal polypeptides (Cociancich *et al.*, 1994; Boman, 1995). Among the antibacterial factors are the cecropins, a family of peptides with 35–39 amino acid residues. The *Drosophila* *Cecropin* locus has been cloned and characterized (Figure 1A) and the expression profile after induction has been described (Kylsten *et al.*, 1990; Samakovlis *et al.*, 1990; Tryselius *et al.*, 1992). The

CecA1 and *CecA2* genes are mainly expressed in the larval and adult fat body, while the *CecB* and *CecC* genes are predominantly expressed during metamorphosis. The *Cec* genes are also expressed in hemocytes and in two *Drosophila* cell lines (Samakovlis *et al.*, 1992). The expression of the *Cec* genes during an immune response is regulated at the level of transcription. Preliminary characterization of the promoter region has shown that 760 bp of *CecA1* upstream region contains all the *cis*-acting elements necessary for fat body-specific expression after induction with lipopolysaccharides (LPS) (Engström *et al.*, 1993). In the proximal upstream region, there is a 40 bp region that is conserved among all four *Drosophila* *Cec* genes (Figure 1B) (Tryselius *et al.*, 1992). Within this 40 bp region, three highly conserved sequence motifs have been identified, here called region 1 (R1), region 2 (R2) and κ B-like motif. It was previously demonstrated that this κ B-like motif serves as a regulatory element in the induction of a reporter gene in transfection assays (Engström *et al.*, 1993). Similar κ B-like motifs have been identified in the upstream region of several inducible genes for antibacterial factors in *Drosophila* (Kappler *et al.*, 1993), *Hyalophora cecropia* (Sun *et al.*, 1991a,b) and *Sarcophaga peregrina* (Kobayashi *et al.*, 1993), indicating that this may be a commonly used sequence motif for the induction of the immune response in insects (reviewed in Faye and Hultmark, 1993).

In the *Drosophila* blood cell line mbn-2 (malignant blood neoplasm-2) (Gateff *et al.*, 1980) the *Cec* genes are activated by the addition of LPS to the culture (Samakovlis *et al.*, 1992). In agreement with this observation, we have identified a κ B binding activity upon stimulation with LPS. Electrophoretic mobility shift assays (EMSAs), using the *CecA1* κ B motif and extracts from the mbn-2 cells, demonstrated that LPS activates a nuclear κ B binding activity called *Drosophila* immunoresponsive factor (DIF) (Engström *et al.*, 1993). This DNA binding activity was suggested to be involved in the activation of the *Cec* genes. Purification of the *Cecropia* immunoresponsive factor (CIF) (Sun and Faye, 1992a,b) and of a κ B binding factor from the flesh fly *S.peregrina* (Kobayashi *et al.*, 1993) have shown that these insect κ B factors share many properties with, and are likely to be related to, the mammalian Rel factors.

In mammals, the transcription factor NF- κ B (Leonardo and Baltimore, 1989) is activated by a variety of stimuli and translocated into the nucleus where it binds to different κ B sites in the promoter regions of different genes. Many of those genes are involved in various immune reactions (for a recent review on NF- κ B, see Baeuerle and Henkel, 1994). NF- κ B and the *Drosophila* morphogen dorsal (dl) belong to a family of DNA binding proteins called the Rel proteins (Steward, 1987; Nabel and Verma, 1993). NF- κ B and dl are retained in the cytoplasm by inhibitory

Fig. 1. The *Cec* locus in *Drosophila*. (A) Restriction map of part of the cloned genomic region with the four *Cec* genes and the *Andropin* (*Anp*) gene is shown. Boxes indicate coding regions, arrows the direction of transcription. The κ B-like motif (filled circle) is present in all four *Cec* genes, the R1 motif (shaded circle) is present in all but the *CecC* gene, and the R2 motif (open circle) is present in the upstream region of all four *Cec* genes. Restriction enzyme sites are B, *Bam*HI; H, *Hind*III; E, *Eco*RI; Hp, *Hpa*I. (B) Conserved sequences in the *Cec* genes. Shown is the *CecA1* sequence, numbers indicate the distances from the transcription start site and capital letters refer to nucleotides conserved in at least three of the four *Cec* genes (Tryselius *et al.*, 1992).

proteins, I κ B (Baeuerle and Baltimore, 1988) and cactus (Geisler *et al.*, 1992; Kidd, 1992), respectively. These inhibitory proteins are also related and contain a series of ankyrin repeats (reviewed in Blank *et al.*, 1992; Nolan and Baltimore, 1992).

Recently, a novel *Drosophila* Rel gene called *dorsal*-related immunity factor, *Dif*, was isolated (Ip *et al.*, 1993). Immunostaining of fat body from larvae revealed that the *Dif* protein is normally localized in the cytoplasm. When larvae were injected with *Escherichia coli*, or damaged by manual puncture, *Dif* was shortly thereafter found predominantly in the nucleus. It was also demonstrated that bacterially expressed *Dif* protein binds to the κ B motif found in the *CecA1* upstream region (Ip *et al.*, 1993). It has, however, not been proven that the *Dif* cDNA-encoded product can activate the target gene *in vivo*. In this study, we demonstrate that the *Dif* gene product activates transcription from the *Drosophila CecA1* promoter in co-transfection assays. This *trans*-activation is dependent on the presence of the highly conserved 40 bp upstream region containing the κ B-like motif. We also show that the presence of a dimer of such a κ B-like motif is sufficient for *trans*-activation of the reporter gene by *Dif*.

It has been proposed that *dl* is involved in the regulation of the *Drosophila* dipterin gene, which encodes another antibacterial protein (Reichhart *et al.*, 1993). We have compared the *trans*-activation of the *CecA1* gene by *Dif* and *dl*. The experimental results show that *Dif* is the more potent activator of the two.

Results

Nuclear κ B binding activity correlates with *Cec* gene expression in *Drosophila* cell lines

Previous studies have shown that *Cec* gene expression can be induced in two different cell lines, the *mbn-2* cells and one isolate of Schneider Line-2 cells (SL2*), but not in another SL2 isolate (SL2) or in *Kc*₍₀₎ cells (Samakovlis *et al.*, 1992). It was also shown that the *Cec* genes were inducible in ~5% of the SL2* cells compared with 60% of the *mbn-2* cells, and that the level of *Cec* mRNA was much higher in extracts of the *mbn-2* than the SL2* cells (Samakovlis *et al.*, 1992). In another series of experiments, it was demonstrated that the *mbn-2* cells contain all necessary *trans*-acting factors for LPS-inducible expression of *Cec-lacZ* constructs in transfection experiments, and a κ B binding activity called DIF was identified in these cells (Engström *et al.*, 1993). It has, furthermore, been suggested that the *Dif* gene-encoded product is identical to or substitutes part of the DIF κ B-binding activity in *mbn-2* cells (Ip *et al.*, 1993). To investigate a possible correlation between the presence of the DIF κ B-binding activity and expression of the *Cec* genes in different *Drosophila* cell lines, we performed EMSAs. A ³²P-labeled oligonucleotide containing the κ B-like motif (*ceckB*) present in the upstream region of the *Cec* genes was used as binding site and analyzed with nuclear and cytoplasmic extracts from unstimulated and LPS-stimulated *mbn-2*, SL2, SL2* and *Kc*₍₁₆₇₎ cells (Figure 2). The *mbn-2* cells contained a strong κ B binding activity after stimulation with LPS, as shown in Figure 2A, lane 3 and Figure 2B, lane 3, and as previously reported (Engström *et al.*, 1993). LPS stimulation also activated a κ B binding activity, with a similar electrophoretic mobility, in SL2* cells (Figure 2A, lanes 7 and 11). In contrast, extracts from the SL2 isolate that does not promote *Cec* gene expression by LPS stimulation did not produce a shifted band (Figure 2A, lanes 5 and 9). The *Kc*₍₁₆₇₎ cell line contained a weak inducible κ B binding activity in cytoplasmic but not in nuclear extracts (Figure 2B, lanes 5 and 7). In addition to the LPS-inducible κ B binding activity, a band shift with higher mobility was produced from most of the extracts, with an especially strong intensity in the *Kc*₍₁₆₇₎ cells. We believe that this DNA-protein complex is unrelated to the activation of the *Cec* genes because it is relatively sequence unspecific and probably consists of a single strand DNA binding activity (L.Kadalayil, unpublished results). These results demonstrate that there is a correlation between the presence of a nuclear κ B binding activity (DIF) and the LPS-inducible expression of the *Cec* genes. Furthermore, the amount of nuclear κ B binding activity (DIF) is much higher in the *mbn-2* cells than in the SL2* cells (for comparison, see Figure 2A, lanes 3 and 7), which nicely corroborates the previous result that the *Cec* genes are induced to a higher level of expression in the *mbn-2* than in the SL2* cell line (Samakovlis *et al.*, 1992). This correlation indicates that the expression of the *Cec* genes involves a κ B binding factor or complex. It was previously shown that the LPS-induced κ B binding activity in *mbn-2* cells contains the *Dif* gene product, since pretreatment of the nuclear extract with *Dif*-specific antiserum abolished the bandshift in an EMSA (Ip *et al.*, 1993). Here we expanded the experiment

Fig. 2. Presence of the κ B binding activity and Dif mRNA in different *Drosophila* cell lines. (A–C) Electrophoretic mobility shift assays using a 32 P-labeled *cec*κB probe 5'-d(TCGAGACACGGGGATTTTGCAC) with uninduced (–) or LPS-induced (+) nuclear (N) and cytoplasmic (C) extracts from (A) mbn-2, SL2 and SL2* cells and (B) mbn-2 and Kc(167) cells. The SL2 and the SL2* cell lines are two different isolates, one expressing the *Cec* genes (SL2*) after stimulation with LPS and other stimuli, while the SL2 isolate does not promote *Cec* gene expression (Samakovlis *et al.*, 1992). (C) LPS-induced nuclear extract from mbn-2 cells preincubated with anti-dl antiserum (lane 2), anti-p65 antibody (lane 3), anti-Dif antiserum (lane 4) and preimmune serum (pi) (lane 5). The κ B binding activity (DIF) is only affected by preincubation with Dif antibodies, while the unrelated band is unaffected by all antibodies. (D) Northern blot analysis. Total RNA was prepared from mbn-2 cells treated with LPS for 1, 8, 16 and 24 h and from untreated (–) mbn-2 and SL2 cells, and from climbing 3rd instar larvae, as indicated. The filter was probed with cDNA of *Dif* and of the constitutively expressed ribosomal protein *Rp49* gene as a control of RNA loading. The normalized ratios of Dif transcript to *Rp49* band intensities are indicated at the bottom of the figure. The molecular weight markers to the right are in units of kilobases.

to also include dl antiserum and an antibody directed against the mammalian NF- κ B p65. As seen in Figure 2C, only the Dif antiserum abolished the κ B binding activity, while pretreatment with the other two antibodies or with preimmune serum had no effect. Therefore, Dif, but not dl, seems to be a component of the κ B binding activity in mbn-2 cells. The weak so-called 'unrelated' bandshift was not affected by any of the sera, indicating that neither Dif nor dl are present in that complex.

We also performed Northern analysis to investigate the presence of Dif mRNA in the mbn-2 and SL2 cell lines and, as seen in Figure 2D, both these cell lines contain Dif mRNA. That the unresponsive SL2 isolate also contains Dif mRNA was surprising, since it lacks nuclear κ B

binding activity, and does not provide expression of the *Cec* genes after LPS treatment. Therefore, there is a correlation between the presence of a nuclear κ B binding activity and the inducibility of the *Cec* genes, but not between the presence of Dif mRNA and expression of the *Cec* genes. The simplest explanation for this is that the unresponsive SL2 isolate lacks some additional activity or component necessary for the formation of an active κ B binding complex and for the induction of the *Cec* genes, although these cells contain Dif mRNA.

The Northern analysis also demonstrated a basal level of constitutive synthesis of the *Dif* gene in the mbn-2 cell line (Figure 2D). Addition of LPS to the mbn-2 cells resulted in a 3-fold increase in the level of Dif mRNA

after 1 h, and then it declined to the same level as in uninduced cells at 16 h after stimulation. A similar result was found in whole flies by Northern analysis of RNA extracted at different time points between 1 and 36 h postinjection of LPS. *Dif* mRNA was expressed in un-injected flies and the levels increased by a factor of two at 3 h postinjection, and then declined (M.Dushay, personal communication). Therefore, *Dif* is a continuously expressed gene which shows a modest increase in the level of transcripts after stimulation with LPS. Whether or not this increase in *Dif* mRNA after LPS stimulation is functionally important, cannot be answered from these results.

We also compared the amount of *Dif* mRNA in *mbn-2* cell extracts with that in total extract from 3rd instar larvae. The *Dif* mRNA in the larval extract was 13 times higher than in the *mbn-2* cell extract, in the absence of LPS stimulation. It was previously found by immunostaining that the strongest *Dif* protein expression was localized to the larval fat body (Ip *et al.*, 1993), and it is therefore likely that most of the larval *Dif* mRNA seen in Figure 2D is from fat body cells. The present data suggest that although the *Dif* gene is expressed in hemocytes, in some cell lines and in larval fat body, other activities/components are also necessary for the formation of an active κB binding complex and for activation of the *Cec* genes.

The *Dif* gene product activates *Cec* gene expression in a sequence-specific manner

In order to test if the cloned *Dif* cDNA encodes a factor that can *trans*-activate the *Cec* genes, we performed co-transfection assays. We analyzed the expression of β -gal from different *Cec-lacZ* fusion constructs in the presence of a *Dif* expression plasmid. The *Dif* cDNA, (Figure 3A) (Ip *et al.*, 1993) was cloned into the *Drosophila* expression vector pAct5C-PL. Co-transfections of pAct-*Dif* with the *Cec-lacZ* reporter plasmid pA10 (Figure 3B), containing the full upstream region of the *CecA1* gene, led to a strong activation of the reporter β -gal expression in *mbn-2* cells (Figure 4A). When increasing amounts of *Dif* expression plasmid were analyzed, the level of reporter gene expression was proportional to the amount of added pAct-*Dif* within the range of 0.5–2 μ g expression plasmid used. The relative β -gal activity was 50–100 times higher in the presence of co-transfected *Dif* than without, i.e. co-transfection of pA10 together with the empty expression vector pAct5C-PL.

During overexpression of *Dif*, by transfection of 1–2 μ g of pAct-*Dif*, the addition of LPS to the transfected cells led to a 30–50% increase in the β -gal activity from the pA10 construct (Figures 4A and 5A). When higher amounts of the pAct-*Dif* expression vector were transfected (6 μ g), the reporter gene activity became saturated and LPS addition did not further stimulate the β -gal expression. This result is in agreement with the conclusion that LPS stimulation of *Cec* gene expression would be mediated via *Dif*.

Overexpression of pAct-*Dif* with pA10 in the SL2 isolate, which contains *Dif* mRNA but no κB binding activity, resulted in a low but significant level of reporter gene expression (Figure 4A). The activation level increased with higher amounts of transfected expression plasmid. In

Fig. 3. (A) Schematic drawing of the *Dif* cDNA. The line refers to the *Dif* cDNA sequence, the box to the coding sequence, the hatched box to the Rel domain and the filled box to a glutamine-rich region present in the C-terminal part of the *Dif* protein (Ip *et al.*, 1993). To create the pAct-*Dif* expression plasmid, the *Dra*I fragment, which encodes the entire open reading frame of 667 amino acids, was subcloned into the expression plasmid pAct5C-PL (Krasnow *et al.*, 1989). The truncated Δ *Dif*-531 protein lacks the C-terminal 137 amino acids and contains the coding sequence from the N-terminal end to the *Hpa*I site in the cDNA, plus one extraneous leucine residue. (B) Drawing of the *Cec-lacZ* reporter plasmids used in this study. The constructs contain upstream untranslated regions of the *CecA1* gene, including the transcription start site (arrow) fused to an SV40 leader (filled box), providing a translational start site in-frame with the *E. coli lacZ* coding sequence (hatched box). Numbers refer to positions relative to the cap site. Construct p(*ceckB*)₂ contains a dimer of the κB -like motif found upstream of the *Drosophila CecA1* gene, while the p(*ceckBmut*)₂ contains a dimer of a mutated κB motif, both inserted at -68 in pA11. Constructs p(*attkB*)₃ and p(*attkBmut*)₃ contain a trimer of the *attacin* κB -like motif or a mutated version of this motif from *H. cecropia*, inserted at -68 in pA11 (Engström *et al.*, 1993). The presence of the R1, κB -like and R2 motifs in the different constructs is indicated.

Fig. 4. Dif is a sequence-specific *trans*-activator of the *CecA1* promoter. (A) Relative β -gal activity in mbn-2 or SL2 cell extracts after co-transfection of 1 μ g of the *Cec-lacZ* reporter plasmid pA10 (see Figure 3B) with increasing amounts of the pAct-Dif expression plasmid (see Figure 3A). The total amount of expression vector was kept constant in all samples (6 μ g) by the addition of the pAct5C-PL vector. Using this high amount of expression vector resulted in ~ 10 times lower β -gal activity compared with transfections using 1 μ g of expression vector, probably because of exhaustion of the basal transcription machinery. Unstimulated cells (open rectangles) or LPS-stimulated cells (filled rectangles). The data shown are averages of at least three independent transfection experiments. (B) Relative β -gal activity in mbn-2 cells after overexpression of Dif protein by co-transfection of 1 μ g of the pAct-Dif expression plasmid (shaded bars) and 1 μ g of different *Cec-lacZ* reporter plasmids (see Figure 3B), as indicated. The empty bar shows the level of pA10 reporter gene expression in mbn-2 cells without overexpression of Dif but in the presence of 1 μ g of the empty expression vector pAct5C-PL. The cells were incubated with LPS (10 μ g/ml) for 4 h prior to harvest to obtain full activation of the immune response. The data shown depict averages of at least three independent experiments.

comparison with the activation in the mbn-2 cells, however, the maximum level of β -gal expression did not reach higher than 10% of the highest levels of *trans*-activation in the mbn-2 cell line. This pronounced difference in expression of the *Cec-lacZ* reporter construct cannot be explained solely by the presence of endogenous κ B binding activity in the mbn-2 cells. Instead, it supports the suggestion that the mbn-2 cells, but not this SL2 cell isolate, contain some component(s) or activity(ies) that is necessary for the formation of an active κ B binding complex, even in the presence of high amounts of overexpressed Dif, and for the induction of the *Cec* genes.

In summary, the strong activation of *Cec-lacZ* expression in the mbn-2 cells and the correlation between the amount of transfected Dif with the levels of reporter gene expression identify Dif as a limiting factor for *Cec* gene

Fig. 5. Overexpressed dl protein has a dominant negative effect on Dif *trans*-activation. (A) Relative β -gal activity in mbn-2 cell extracts after overexpression of Dif or dl proteins by co-transfection of 1 μ g of one of the expression plasmids pAct-Dif, pAct5C-dl or pAct5C-PL with 1 μ g of the respective *Cec-lacZ* reporter plasmids (see Figure 3B), as indicated. The cultures were incubated with (shaded bars) or without (open bars) LPS (10 μ g/ml) prior to harvest. The data shown depict average values from three or more independent experiments. (B) Relative β -gal activity in mbn-2 cell extracts after transfection of pAct-Dif and pAct5C-dl expression plasmids in the amounts indicated at the bottom, with 1 μ g of the pA12 *Cec-lacZ* reporter plasmid. The total amount of expression plasmid was 1 μ g in each sample. The broken line indicates the expected additive β -gal values, by Dif and dl *trans*-activation separately. The actual β -gal activity is lower than that expected during co-transfection, suggesting that Dif and dl proteins negatively interfere with each other. The data shown depict the average values of three independent transfection experiments. All extracts were treated with LPS (10 μ g/ml) prior to harvest.

expression in transfected mbn-2 cells. It does not rule out the possibility that another factor(s), already present in sufficient amounts in mbn-2 cells, activates *Cec* gene expression cooperatively with Dif. In addition, our experiments demonstrate that different cell lines may carry

endogenous or exogenous *Dif* mRNA and protein without being able to activate *Cec* gene expression efficiently.

To analyze whether the *trans*-activation of the *CecA1* gene by the *Dif* gene product is sequence specific, co-transfections were performed with reporter constructs carrying different parts of the *CecA1* upstream region, all fused to the *lacZ* gene (Figure 3B). Co-transfections of pAct-*Dif* with the reporter plasmid pA12, a 5' deletion construct that contains 105 bp of the upstream region including the R1, κ B-like and R2 motifs (see Figures 1B and 3C), resulted in higher levels of β -gal activity than with pA10 (Figures 4B and 5A). This result narrows down the necessary region for *Dif trans*-activation to the parts present in pA12. Furthermore, it indicates that the upstream region between -105 bp and -760 bp contains negatively acting *cis* elements, repressing the *trans*-activation by co-transfected *Dif*.

Co-transfections of the *Dif* expression plasmid with the reporter plasmid pA11 (Figure 3B), a deletion construct carrying only 68 bp of the *CecA1* upstream region, resulted in a β -gal activity much lower than from the pA12 reporter, identifying the region between -111 and -68 as being crucial for the *trans*-activation by *Dif*. The importance of this region was investigated further by analyzing expression from the reporter plasmid pA15, which has an internal deletion between -105 and -69, removing the R1 and the κ B-like motif (Figures 1B and 3B). The β -gal expression from pA15 in the presence of co-transfected *Dif* was very low (Figure 4B), demonstrating the requirement for either the R1 or the κ B motif, or both, for the *trans*-activation by *Dif*.

To test directly if the κ B motif is sufficient for *Dif trans*-activation, we analyzed the expression from reporter plasmids carrying multimerized κ B-like motifs (Figure 4B). Dimers of the κ B-like motif called *ceckB*, as well as of a mutated version called *ceckBmut*, were inserted into the minimal reporter construct pA11 (Figure 3B). Co-transfection of pAct-*Dif* with the p(*ceckB*)₂ construct resulted in the highest level of reporter gene expression in our series of experiments (Figure 4B). The reporter gene activity from p(*ceckB*)₂ was ~10 times higher than the activity from pA11. In contrast, the p(*ceckBmut*)₂ construct was not significantly activated by *Dif*, demonstrating the requirement for an intact κ B motif for activation by *Dif*. Next, we analyzed trimers of the κ B-like motif present in the upstream region of the *Drosophila* dipterin gene and of the *H. cecropia* attacin, cecropin and lysozyme genes, called the *attkB* motif, and of the respective mutant, called *attkBmut*. Co-transfections of pAct-*Dif* with p(*attkB*)₃ and p(*attkBmut*)₃ did not lead to any activation of the reporter gene. This result was surprising since it has been shown previously that the p(*attkB*)₃ can mediate activation in transfection assays in *mbn-2* cells, without co-transfection of *Dif* (Engström *et al.*, 1993). Activation of p(*attkB*)₃ in the absence of *Dif* overexpression was evident in this series of experiments as well (data not shown), but there was no superactivation by the overexpression of *Dif*. This result could indicate that *Dif* only activates *ceckB*-containing promoters, like the *Drosophila Cec* genes, and not *attkB*-containing promoters, like the *Drosophila* dipterin gene. Promoters of the latter type would then probably be activated by another Rel family member or heterodimers thereof. The p(*attkB*)₃

reporter construct was, however, not *trans*-activated by co-transfected pAct-*dl* in a parallel experiment (data not shown). Another explanation is that *trans*-activation of *attkB*-containing promoters requires cooperation between *Dif* and another factor(s). Such a factor(s) is probably normally present in the *mbn-2* cells in sufficient amounts, since the p(*attkB*)₃ was activated in transfection assays (Engström *et al.*, 1993), but becomes limiting during *Dif* overexpression. Most importantly, however, our results clearly demonstrate that *Dif* is a sequence-specific transcription factor, which efficiently *trans*-activates promoters containing the *ceckB* motif. Therefore, it is very likely that *Dif* is a major regulatory factor in the induction of *Cec* gene expression during an immune response, and that the *ceckB* motif is necessary for this *trans*-activation.

Dif is a more potent and sequence-specific trans-activator of CecA1 than is dl

It has been proposed that the transcription factor *dl* is involved in the regulation of the *Drosophila* dipterin gene (Reichhart *et al.*, 1993). *Dif* and *dl* were previously tested by EMSA for their ability to bind to the *ceckB* motif present in the 5' end of the *Cec* genes, and this demonstrated that the *Dif* protein has a substantially higher binding affinity for the *ceckB* motif than does the *dl* protein (Ip *et al.*, 1993). Furthermore, the preincubation of LPS-induced nuclear extracts with anti-*Dif* antibodies, but not with anti-*dl* antibodies, abolished the nuclear binding activity to the *ceckB* motif, strongly indicating that the LPS-induced nuclear κ B binding activity in *mbn-2* cells contains *Dif* but not *dl* protein (Figure 2C). It has not been tested directly, however, whether *dl* can activate transcription from the *Cec* promoters. To answer this question, we performed co-transfections of a *dl* expression plasmid, pAct5C-*dl* (wt) (Rushlow *et al.*, 1989) with the *CecA1-lacZ* reporter genes. To directly compare the *trans*-activation of the *CecA1* gene by *Dif* and *dl*, we performed the co-transfection experiments using the different *CecA1-lacZ* constructs in parallel (Figure 5A). The constructs were made in the same expression vector. The *trans*-activation was 3–4 times higher by transfected *Dif* than by transfected *dl*, when the pA10 and pA12 reporter constructs were analyzed (Figure 5A). The *trans*-activation by *dl* was less influenced by different upstream regions, as pA10, pA11 and pA12 gave similar results. Furthermore, the pA15 construct was expressed at a somewhat higher level in the presence of overexpressed *dl* than *Dif*, indicating that *dl* is less sequence specific than *Dif* in its action on the *CecA1* promoter. It has previously been reported that *dl* can act as a relatively unspecific transcription factor in co-transfection experiments, possibly by interacting directly with the basal transcription machinery (Norris and Manley, 1992), or by interacting with low affinity binding sites in the vector (Thisse *et al.*, 1991). Furthermore, it was reported recently that expression of the *Drosophila* dipterin and cecropin A genes are not affected in *dl*¹/*dl*¹ background, indicating the non-essential role of *dl* for the expression of immune related genes (Lemaitre *et al.*, 1995). In conclusion, both *Dif* and *dl* can *trans*-activate *CecA1* expression, but *Dif* is clearly more efficient than *dl*, and *Dif* shows a greater preference and specificity for this promoter than does *dl*. This means that *Dif* is the more important activating factor of *Drosophila Cec* genes *in vivo*.

The co-transfections with pAct5C-*Dif* and pAct5C-*dl* were also analyzed with and without LPS stimulation of the cells (Figure 5A). The reporter gene activity was stimulated by LPS addition in the presence of overexpressed *Dif* but not *dl*, indicating that an LPS effect can be mediated via *Dif* but not via *dl*. As mentioned above, the LPS stimulatory effect was specific for the pA10 construct, and not seen with the pA12 construct, although pA12 contains the κ B motif (Figures 4A and 5A). These results may indicate that negatively acting elements in the distal upstream region have a function in repressing the *CecA1* gene expression in the absence of stimulus from bacterial products. Only pA10 would then provide the *cis*-acting elements for both positive and negative regulation.

The *dl* protein has a dominant negative effect on *Dif* trans-activation

Having shown that overexpressed *dl* protein can also activate *CecA1* gene expression, though to a lesser extent than *Dif*, we analyzed possible synergistic interactions between *Dif* and *dl* proteins during *trans*-activation, which might lead to higher expression. The two expression plasmids were mixed in different relative amounts and co-transfected with the pA12 reporter plasmid. As demonstrated in Figure 5B, the *trans*-activation by pAct-*Dif* was negatively affected by the addition of pAct5C-*dl*. The β -gal expression was lower than expected if it resulted solely from the additive activation by *Dif* and *dl* protein in the cells, as is indicated by a broken line in Figure 5B. Therefore, the expression of *dl* protein has a dominant negative effect on *Dif* *trans*-activation of the *CecA1* gene. Several explanations for this result could be suggested, one being that *Dif* and *dl* proteins form heterodimers that possess weaker DNA binding and/or *trans*-activation capacity than *Dif* does by itself, as a monomer or homodimer. Another explanation could be that *dl* and *Dif* proteins do not interact directly but compete for interaction with another factor or activity in the cell.

The C-terminal end of *Dif* constitutes part of a *trans*-activation domain

It was previously shown by EMSA, using a set of bacterially expressed C-terminal truncated forms of *Dif* protein, that the Rel domain is responsible for DNA binding (Ip *et al.*, 1993). To begin a mapping of other functional domains of the *Dif* protein, we constructed a C-terminal truncation of *Dif* (Δ *Dif*) by inserting a stop codon at the *HpaI* site in the coding region of *Dif* in the pAct5C expression plasmid (see Figure 3A). This produces a protein lacking the C-terminal 137 amino acids, a truncation that does not affect DNA binding significantly (Ip *et al.*, 1993). The deletion removed, however, a glutamine/proline-rich region, previously suggested to have a function as a transcriptional activation domain (Ip *et al.*, 1993), according to the functional role of glutamines, prolines and hydrophobic residues in activation domains of known transcriptional activators (Mitchell and Tjian, 1989). Using a *Dif* antiserum in Western blot analysis, we found that both the transfected full length pAct-*Dif* and the truncated pAct- Δ *Dif* constructs produced proteins of the expected size (see Figure 6A). The *Dif* antiserum gave a very weak band at the expected size of *Dif* in the control lane of untransfected mbn-2 cells. Instead, a band

Fig. 6. The 137 residue C-terminus of the *Dif* protein is required for full *trans*-activation of the *Cec-lacZ* reporter genes. (A) Western blot analysis. Whole cell extracts were prepared from mbn-2 cells, untransfected or transfected with pAct-*Dif* or pAct- Δ *Dif*-531 (Figure 3A). The amount of loaded protein of each of the extracts is indicated at the top. The molecular weight markers to the right are in units of kDa. (B) Relative β -gal activity in mbn-2 cell extracts after co-transfection of 1 μ g of the pA10 reporter plasmid (Figure 3B) with pAct-*Dif* or the truncated version pAct- Δ *Dif*-531 expression plasmids (Figure 3A), or with the vector pAct5C-PL, as indicated. The transfected cells were incubated without (open bars) or with (shaded bars) 10 μ g/ml LPS for 4 h prior to harvest. The β -gal activity data shown depict average values from four independent experiments. (C) Relative β -gal activity in mbn-2 cell extracts after co-transfection of 1 μ g of the pA12 reporter plasmid (Figure 3B) with pAct-*Dif* and the truncated version pAct- Δ *Dif*-531 expression plasmids (Figure 3A), in the amounts indicated at the bottom. All cells were incubated with 10 μ g/ml LPS for 4 h prior to harvest. The β -gal activity data shown depict average values from four independent experiments.

at ~65 kDa is present, which may be a modified form of Dif. The Dif antiserum also gave a strong band at ~116 kDa, which may either be crossreaction with a non-related protein or possibly with another Rel protein (see Discussion).

Co-transfections of Δ Dif and the pA10 *Cec-lacZ* reporter construct (see Figure 6B) demonstrated that Δ Dif retained only ~30–40% of its *trans*-activation capacity. Therefore, the C-terminal end, which contains the glutamine/proline-rich region, is important for *trans*-activation and constitutes at least part of a *trans*-activation domain. The effect of LPS stimulation was lower with the truncated form of the Dif protein (Figure 6B), suggesting that the C-terminal end also may be important for the mediation of induction by bacteria. Mixing full length Dif with Δ Dif in the same co-transfection experiment (Figure 6C) gave a linear correlation between the amount of full length Dif and the level of reporter gene activity, showing no tendency of a dominant negative effect by Δ Dif on full length Dif function.

Subcellular localization of Dif in hemocytes and cell lines

The activity of the Rel family of transcription factors is regulated at the level of nuclear translocation (Baeuerle and Henkel, 1994). It was demonstrated that Dif and dl proteins are normally located in the cytoplasm in larval fat body and, after injury or infection, are found in the nucleus (Ip *et al.*, 1993; Reichhart *et al.*, 1993). We have investigated the intracellular localization of Dif protein in larval hemocytes, and of Dif and dl proteins in different cell lines, using Dif- and dl-specific antisera. Dif immunostaining of hemocytes, collected from climbing 3rd instar larvae, demonstrated that Dif protein is present *in vivo* in hemocytes (Figure 7A). In small hemocytes, the plasmatocytes and podocytes (Rizki, 1978), Dif staining was uniform throughout the cell. Injection of LPS into the larvae prior to dissection and collection of hemocytes led to an enrichment of Dif staining in the nucleus (Figure 7C). This nuclear enrichment was not complete; all cells still revealed Dif staining in the cytoplasm. In large hemocytes, the lamellocytes (Rizki, 1978), the nuclear Dif staining was stronger than the cytoplasmic staining, both before and after LPS treatment of the larvae. Since the cytoplasm is very large and flat in lamellocytes, the amount of Dif protein may, however, be relatively the same in the cytoplasm and the nucleus. After LPS treatment of the larvae, the nuclear staining in lamellocytes became even more intense in comparison with the cytoplasmic staining (data not shown). Our conclusion is that Dif protein is present in larval hemocytes, both in the nucleus and cytoplasm, and that LPS injection into larvae leads to an enrichment of the nuclear localization of Dif protein.

Immunostaining of mbn-2 cells with Dif and dl antibodies revealed that Dif staining was more intense than dl staining in this cell line (Figure 7E and F). Dif was detected both in the cytoplasm and in the nucleus of most of the uninduced cells, either as a uniform staining throughout the cell (arrow) or with a stronger cytoplasmic staining (Figure 7E). Addition of LPS to mbn-2 cells did not result in a striking difference of Dif localization, and we did not detect any cells in which the anti-Dif staining was exclusively nuclear. Parallel experiments were also

performed in the SL2 cells with the same result (data not shown).

Immunostaining of mbn-2 cells after overexpression of Dif protein by transfection of pAct-Dif expression plasmid led, as expected, to very strong staining with the antibody. The transfected full length Dif was present both in the cytoplasm and the nucleus, but the staining was stronger in the cytoplasm (Figure 7G). A parallel experiment verified the specificity of the Dif and dl antisera used in this study, as the Dif antiserum did not crossreact with transfected dl protein, and vice versa (data not shown).

In a previous experiment, Rushlow *et al.* (1989) demonstrated that truncations in the C-terminal part of the dl protein (Δ dl-561) lead to an exclusively nuclear localization of the protein in SL2 cells, suggesting an important function of the C-terminal part for cytoplasmic retention and for interactions with the inhibitory cactus protein. We analyzed, in a similar experiment, the subcellular localization of Δ Dif-531 and Δ dl-561 after transfection in the mbn-2 cell line. Overexpressed Δ dl-561 localized exclusively in the nucleus in the transfected mbn-2 cells (see Figure 8B). Overexpressed Δ Dif-531 gave a strong staining in both the nucleus and the cytoplasm, as seen in Figures 7H and 8A. The staining pattern was complex and, in order to study the localization in greater detail and to focus on intranuclear structures, we used confocal immunofluorescence microscopy. As illustrated in Figure 8A, which is a composite image of consecutive optical sections through the nucleus, the staining was intense in the nuclear membrane with trabecular structures penetrating the nuclear space. The nature of these structures is not known but may possibly constitute parts of the nuclear scaffold. The cytoplasmic Dif staining was also strong. Our conclusion is that truncation of the 137 C-terminal residues of Dif did not lead to an exclusive nuclear localization, but interfered with the normal localization, and possibly with the transport of the protein through the nuclear pores, as it seems that the Δ Dif-531 protein was attached to or trapped in the nuclear membrane.

Discussion

Dif mRNA and protein are expressed in several *Drosophila* cell lines and in larval hemocytes

We have used several different methods to analyze for the presence of Dif mRNA, Dif protein and a κ B binding activity called DIF, in *Drosophila* cells. It is evident that the nuclear κ B binding activity, DIF, is present in high amounts after immune induction in mbn-2 cells, at lower amounts in a SL2* isolate, but absent in another SL2 isolate and in Kc₍₁₆₇₎ cells (see Figure 2A and B). This result correlates very well with previous results which demonstrated a high level of *Cec* gene expression upon LPS stimulation in mbn-2 cells, a low level of expression in SL2* cells and no expression in SL2 and Kc₍₀₎ cells (Samakovlis *et al.*, 1992). This strong correlation between DIF nuclear κ B binding activity and the expression of the *Cec* genes is suggestive of a role for DIF in the induction of *Cec* gene expression upon LPS stimulation. The DIF nuclear κ B binding activity in mbn-2 cell extracts was sensitive to treatment with Dif, but not with dl, antibodies, indicating that Dif but not dl is a component of DIF.

Northern blot analysis demonstrated that the mbn-2

Fig. 7. Intracellular localization of Dif and dl proteins. (A–D) Dif immunostaining of hemocytes from 3rd instar climbing larvae. (A) and (C) immunofluorescence, (B) and (D) differential interference contrast micrograph. In hemocytes from untreated larvae (A, B), the Dif staining was uniform throughout the cells. In hemocytes from larvae injected with LPS (10 µg/ml) 30 min prior to dissection and collection (C, D), the Dif staining was predominantly nuclear. (A–D) were done in parallel and exposed equally. The bar in (A) is 5 µm and shows the magnification of (A–D). (E and F) Immunostaining of mbn-2 cells using a rat anti-Dif antiserum (E) or rat anti-dl antiserum (F). Most cells displayed Dif staining in both the nucleus and cytoplasm, either uniformly throughout the cell (arrow) or with a stronger cytoplasmic staining. (G and H) Overexpression of Dif protein (G) and of the truncated Δ Dif-531 protein (H) and immunostaining with rat anti-Dif antiserum. (E–H) were done in parallel and photographed in the same way except that the transfected cells (G, H) were exposed for a shorter time than those of the untransfected cells (E, F). The bar in (E) is 5 µm and shows the magnification of (E–H). Incubation of mbn-2 cells with the FITC-conjugated secondary rabbit anti-rat antibody without prior incubation with the Dif or dl antisera did not lead to any detectable fluorescence staining (data not shown)

cells and, to our surprise, also the unresponsive SL2 cells, express Dif mRNA. Both these cell lines also contain Dif protein, according to the immunostaining experiments using Dif antibodies. We conclude that the presence of Dif mRNA and protein is not linked directly to the formation of a κ B binding activity, even after stimulation by LPS, without the involvement of some additional

activity or components, which probably are missing in the unresponsive cell lines.

Dif mRNA is present in late embryos, larvae and flies even without stimulation of an immune response (Ip *et al.*, 1993), and it has now been demonstrated that LPS stimulation in the mbn-2 cells (this work) and in flies (M.Dushay, personal communication) leads to a maximal

Fig. 8. Subcellular localization of truncated Dif and dl proteins. Overexpression of truncated forms of Dif and dl proteins in mbn-2 cells by transfection of (A) pAct- Δ Dif-531 (Figure 3A) or (B) Δ dl-561 (Rushlow *et al.*, 1989) followed by incubation with anti-Dif (A) or anti-dl (B) antisera, and visualized by immunofluorescence confocal scanning microscopy. The photographs show composite images of consecutive sections through the nuclear space; five sections of 0.3 μ m each (A) and six sections of 0.25 μ m each (B).

3-fold increase in the amounts of Dif mRNA. From these data, one can conclude that the *Dif* gene is constantly expressed after onset during late embryogenesis, and that induction of target gene expression by Dif protein is most likely the result of posttranslational activation rather than induction of transcription of the *Dif* gene. That Dif is a continuously expressed factor was also confirmed by immunostaining with Dif antibodies, which showed that Dif protein is present in larval fat body (Ip *et al.*, 1993), in mbn-2 and SL2 cells, and in larval hemocytes prior to LPS activation.

Interactions between Dif and other transcription factors

It has been shown in several studies that the mammalian Rel factors and also dl protein act as dimers (Govind *et al.*, 1992; Baeuerle and Henkel, 1994). Our results demonstrate that dl protein has a dominant negative effect on Dif *trans*-activation. This may have several explanations, but the most obvious one would be that Dif and dl proteins form heterodimers. In EMSAs, bacterially expressed dl protein does not bind the ceckB motif (Ip *et al.*, 1993; L.Kadalayil, unpublished data). If Dif/dl heterodimers interact only poorly with the ceckB motif that could explain the dominant negative effect by dl protein on Dif *trans*-activation in co-transfection assays.

Another series of co-transfection experiments was performed by overexpressing full length Dif and truncated Δ Dif-531 together (see Figure 6C). There was no dominant negative effect on Dif *trans*-activation by the expression of Δ Dif-531, suggesting either that the two forms do not dimerize or that Δ Dif-531 does not affect DNA binding or *trans*-activation of full length Dif within a Dif/ Δ Dif heterodimer. In this experiment, the binding affinity of a Dif/ Δ Dif heterodimer for the ceckB site is likely to be the same as for the respective homodimers, since it has been shown in EMSAs that the Δ Dif-531 form has a similar κ B binding affinity to that of the full length form of the protein (Ip *et al.*, 1993). Therefore, the decrease in

reporter gene activity is a result of poor *trans*-activation capacity of Δ Dif-531 rather than of affected DNA binding. The results are in agreement with the idea that Dif acts as a homodimer capable of forming heterodimers with dl and possibly also with other putative Rel proteins.

Transfection experiments in mbn-2 cells with a reporter construct containing a trimer of the attkB-like motif inserted into the minimal promoter construct pA11 previously resulted in the same level of reporter gene activity as from the full length *CecA1* construct pA10 (Engström *et al.*, 1993). Therefore, the mbn-2 cells must contain all the factors needed for activation of the p(attkB)₃ construct. The present study shows that overexpression of Dif by co-transfection did not result in any superactivation of the p(attkB)₃ reporter gene, however. This may indicate either that promoters containing the attkB motif (e.g. the *Drosophila* dipterecin promoter) are not activated by Dif or that such promoters are activated by a combination of Dif and other factor(s). It is then assumed that such a factor(s) becomes limiting in relation to the overexpressed level of Dif protein in co-transfection experiments.

In mammals, Rel proteins form both homo- and heterodimers with different binding affinities to different κ B sites in various genes (Grimm and Baeuerle, 1993). If a family of Rel proteins exists also in *Drosophila*, it is reasonable to believe that these proteins would form homo- and heterodimers in a similar manner with different affinities for different κ B motifs. Evidence for the presence of additional Rel proteins in *Drosophila* comes from the recent isolation of a cDNA encoding a novel Rel-containing protein (M.Dushay, B.Åsling and D.Hultmark, personal communication). Whether Dif and this newly identified Rel protein interact will be an important question to address in future experiments. Another possibility is that Dif interacts with other non-Rel proteins during *trans*-activation of some promoters. It has been shown that mammalian NF- κ B physically associates with bZIP proteins like the NF-IL6 transcription factor (LeClair *et al.*, 1992), the bZIP transactivator of Epstein-Barr virus

(BZLF1) (Gutsch *et al.*, 1994) and with other C/EBP family members (Stein and Baldwin, 1993; Stein *et al.*, 1993). Recently, it was also shown that activation of the promoters of both the human β -interferon and the human E-selectin genes by NF- κ B requires cooperation by the high mobility group protein, HMG I(Y) (Thanos and Maniatis, 1992; Whitley *et al.*, 1994).

This study provides evidence that Dif is a potent *trans*-activator of the *Drosophila* *Cec* genes, and that overexpressed Dif is very sequence specific in its action. It is unlikely, however, that Dif would only activate the *Cec* genes and not be utilized by the organism for the activation of several different immune related genes. Future experiments should identify the target genes for Dif activation, and whether Dif, in combination with other factors, is able to activate promoters with alternative κ B motifs.

Regulation of Dif activity

The activity of the Rel proteins, like NF- κ B and dorsal, is regulated at the level of nuclear transport. For NF- κ B, a number of different stimuli lead to dissociation of the inhibitor I κ B α and the subsequent nuclear translocation of the active NF- κ B molecule (reviewed in Baeuerle and Henkel, 1994). In a similar way, the dl protein responds to a signal transduction pathway via the activation of the Toll receptor, through the gene products of *pelle* and *tube*, leading to dissociation of the dl/cact complex and nuclear localization of dl protein in the ventrally located nuclei (Govind and Steward, 1991). Is Dif regulated through a similar pathway to NF- κ B or dl, or a combination of both? It was previously shown that, in the larval fat body, Dif protein is normally localized in the cytoplasm but, after injection of LPS, the protein is enriched in the fat body nuclei (Ip *et al.*, 1993). The immunolocalization of Dif in hemocytes and cultured cells, using the same anti-Dif antiserum as in the previous study of fat body, did not reveal such a dramatic difference in the localization of Dif before and after LPS treatment. Induction with LPS did result in some enrichment of Dif in the nuclei of larval hemocytes, but it was less apparent in cultured cells. In all cases, Dif was present in the nuclei prior to LPS incubation. The κ B binding activity in mbn-2 cells (see Figure 2A and B), as well as the expression of the *Cec* genes, requires, however, stimulation with LPS or with other inducers of the immune response, although these cells clearly contain Dif protein in their nuclei in the absence of stimuli. This may imply that the mechanism of Dif activation is partly different in hemocytes from that in fat body, and/or that nuclear translocation and activation of Dif are two independent processes. In this model, the nuclear localization of Dif would occur without LPS stimulation in hemocytes and mbn-2 cells. Activation of Dif, according to our results, which would be required in fat body, hemocytes and cell lines, could be achieved at several different hypothetical levels: dissociation of a nuclear inhibitor protein, modification of the Dif protein itself or removal of a transcriptional repressor from the target gene promoter.

Independent evidence suggesting that nuclear translocation and activation of Dif are separate processes comes from experiments with flies and larvae carrying mutations in the dorsoventral signaling pathway. In *Toll*^{10b} larvae,

the mutant form of the Toll receptor is constitutively active, which leads to nuclear localization of Dif protein, as well as to an increase in the level of Dif mRNA (Ip *et al.*, 1993). In these larvae, the expression of the *Cec* genes is not activated without injection of LPS, indicating that nuclear localization of Dif is not sufficient for the activation of the *Cec* genes *in vivo* (Y. Engström, unpublished data). Similar results were recently published by Lemaitre *et al.* (1995), showing that neither the dipterin nor the *CecA* genes are activated in *Toll*^{10b} larvae. In summary, Dif activity seems to be regulated both at the level of nuclear translocation and at another as yet unidentified level.

Materials and methods

Recombinant DNA

Construction of the vector pBSlac20 and the plasmids pA10, pA11, p(att κ B)₃ and p(att κ Bmut)₃ are described in Engström *et al.* (1993). The 5' deletion pA12 was created by removing the fragment between the *Sal*I site in the polylinker and a *Bst*E II site (at -111) from pA10. Blunt ends were created by using Klenow polymerase and the ends were religated. The internal deletion construct pA15 was created by removing the *Bst*EII-*Sph*I fragment from pA10, followed by religation of the ends after trimming the single-stranded (ss) DNA ends using T4 DNA polymerase. The p(ceckB)₂ and p(ceckBmut)₂ constructs were created by annealing the respective 23 oligomers ceckB 5'-TCGAGAC-ACGGGGATTTTGCAC-3' and ceckBmut 5'-TCGAGACACGTTG-ATTTTGCAC-3' and ligating these unphosphorylated oligomers to the opened *Xho*I site of pGEM-11Zf(-) vector (Promega). After phosphorylation, using T4 kinase, the ends were ligated, thereby creating dimers of the oligomers. The oligodimers were cleaved out using *Bam*HI-*Sal*I digestion, filled in with Klenow polymerase and cloned into pBSA11, previously opened with *Xho*I, ssDNA ends trimmed with T4 DNA polymerase and treated with calf intestinal phosphatase. The promoter region including the ceckB and ceckBmut dimers was sequenced to verify the sequence and orientation of the κ B-like motifs.

The Dif expression plasmid pAct-Dif was constructed by subcloning a 2.3 kb *Dra*I fragment containing the coding region of the Dif cDNA (Ip *et al.*, 1993) into the *Drosophila* expression vector pAct5C-PL (Krasnow *et al.*, 1989). The vector was opened in the polylinker with *Kpn*I and the ssDNA ends trimmed with T4 DNA polymerase before blunt end ligation with the *Dra*I fragment. To generate the pAct- Δ Dif-531 construct, pAct-Dif was cleaved with *Hpa*I, which cleaves the Dif cDNA at position 2155 and in the 3' polylinker of the vector. An *Xba*I linker was inserted into the opened plasmid to generate a stop codon in the correct reading frame. This created a Dif protein of 531 amino acids (Δ Dif-531) including one extraneous leucine residue just before the stop codon. Construction of the dl expression plasmids pAct5C-dl and Δ dl-561 has been described previously (Rushlow *et al.*, 1989).

Cell cultures

Drosophila mbn-2 cells (Gateff *et al.*, 1980) and *Drosophila* SL2 tissue culture cells were grown at 25°C in Schneider's medium (purchased from Nord Cell AB, Stockholm) supplemented with 1 $\times$ Glutamax I (GIBCO) and 5% or 10% fetal calf serum (FCS), respectively. Two different isolates of Schneider cells were used, differing in their capability of turning on the *Cec* genes in response to LPS, one responsive (SL2*) and one unresponsive (SL2) (Samakovlis *et al.*, 1992). The mbn-2, SL2 and SL2* cells are the same isolates as used by Samakovlis *et al.* (1992), while the Kc₍₁₆₇₎, obtained from L. Søndergaard, University of Copenhagen, is a different isolate from Kc₍₀₎. The Kc₍₁₆₇₎ cells were grown at 25°C in D22 medium (Sigma) supplemented with 2% FCS. All media were also supplemented with 50 U/ml penicillin, 50 μ g/ml streptomycin and 50 μ g/ml gentamycin (GIBCO).

Transfection of cells

mbn-2 or SL2 cells (5×10^6) were plated on 5 cm dishes in 5 ml complete Schneider's medium. Twenty four hours later, the cells were transfected, using the calcium phosphate precipitation method (Rio and Rubin, 1985). 0.5–6 μ g of expression plasmid (pAct-Dif or pAct5C-dl) was mixed with pAct5C-PL vector DNA so that the amount of expression plasmid was kept constant within each experiment. The expression

plasmid DNA was mixed with 1.0 µg of one of the reporter plasmids, 2 µg of transfection efficiency control plasmid pAcCAT [constructed by K.Furukubo-Tokunaga, described in Engström *et al.* (1993)] and carrier plasmid DNA to 20 µg of total DNA per transfection. The cells were incubated with the precipitate for 16–24 h at 25°C, washed carefully with serum-free Schneider's medium and finally fed with 5 ml of complete medium and incubated at 25°C for 24–48 h. An immune response was activated by the addition of purified LPS (10 µg/ml) from *E.coli* strain 055:B5 4 h before harvesting. Extraction and enzymatic assays were performed exactly as described by Engström *et al.* (1993).

Northern blot analysis

Total RNA was prepared as described by Klemenz *et al.* (1985) from 5×10^7 untreated or LPS-treated mbn-2 cells, SL2 cells or from larvae. 15 µg of RNA was resolved by electrophoresis in a denaturing formaldehyde gel, blotted overnight to a Hybond-N membrane (Amersham), and crosslinked to the membrane with UV light. The cDNA probes of *Dif* and the ribosomal protein gene *Rp49* were radioactively labeled by using the Multiprime DNA labeling system (Amersham) and [32 P]dCTP. Hybridization was performed at 68°C overnight in $6 \times$ SSC ($1 \times$ SSC is 150 mM NaCl and 15 mM sodium citrate), $2 \times$ Denhardt's reagent ($1 \times$ Denhardt's reagent is 0.2 mg/ml Ficoll, 0.2 mg/ml polyvinylpyrrolidone and 0.2 mg/ml bovine serum albumin), 0.1% sodium dodecylsulfate (SDS) and 100 µl denatured herring sperm DNA (10 mg/ml). Final washes were done in $0.2 \times$ SSC, 0.1% SDS at 68°C for 1 h (Sambrook *et al.*, 1989). The filters were exposed to a phosphor screen and the bands were scanned and quantified with a PhosphorImager (Molecular Dynamics).

Western blot analysis

Whole cell extracts were prepared from 5×10^6 mbn-2 cells, untransfected or transfected with pAct-*Dif* or pAct- Δ *Dif-531*. The cells were washed twice in phosphate-buffered saline (PBS) pH 7.3, pelleted and dissolved in 3 volumes of 20 mM HEPES pH 7.9, 0.56 M KCl, 0.2 mM EDTA, 1.5 mM MgCl₂, 2 mM DTT and 25% glycerol. The cells were homogenized by freezing on liquid N₂ and thawing on ice while pipetting up and down 10 times. The lysates were left at 4°C for 1 h, then the pipetting was repeated and finally the lysates were centrifuged to remove cell debris. Cell extract containing 15 µg and 30 µg of protein was separated by electrophoresis in a 7.5% SDS-polyacrylamide gel at constant current of 20 mA for 90 min. The proteins were transferred to a Hybond-C membrane (Amersham) by electrophoresis in a Trans-blot Cell (BIO-RAD), at 200 mA overnight at 4°C (Towbin *et al.*, 1979). The membrane was blocked with 5% low fat milk powder in 0.9% NaCl and 100 mM Tris-Cl pH 7.5, for 2 h and then incubated with rat anti-*Dif* antibody (1:1500) for 2 h. Incubation with alkaline phosphatase-conjugated goat anti-rat antibodies (Sigma), washing and development of the alkaline phosphatase reaction were carried out according to Blake *et al.* (1984).

Electrophoretic mobility shift assay

Nuclear and cytoplasmic extracts were prepared from 10^7 uninduced or LPS-induced cells as described by Grant *et al.* (1992). A synthetic oligonucleotide containing the cecKb sequence 5'-d(TCGAGAC-ACGGGGATTTCAC) was radioactively labeled by using Klenow DNA polymerase and [32 P]dCTP. Unincorporated nucleotides were removed on a NENSORB-50 column (Du Pont). Binding reactions were carried out by mixing 1 ng of 32 P-labeled cecKb probe, 10 µg of nuclear or cytoplasmic extracts and 1 µg of poly(dI-dC) in 20 µl EMSA buffer (100 mM NaCl, 15 mM HEPES, 0.75 mM EDTA, 1 mM DTT and 4.25% glycerol). After incubation at room temperature for 15 min, the EMSA was carried out. The gel was prerun at 11 V/cm for 60 min at room temperature in 22.5 mM Tris borate, pH 8.0, 22.5 mM boric acid, 0.5 mM EDTA. Electrophoresis was carried out under the same conditions for 90 min according to Sun *et al.* (1991b). The gel was dried and exposed to a phosphor screen and scanned with a PhosphorImager (Molecular Dynamics).

Extracts treated with antiserum or preimmune serum were preincubated with 0.5 µl of the respective antiserum for 10 min at room temperature prior to the addition of the probe. The antibodies used were rat anti-*Dif* (Ip *et al.*, 1993), rat anti-dl (Rushlow *et al.*, 1989) and rabbit anti-p65 raised against a peptide mapping with the amino-terminal Rel region of human NF-κB p65 (purchased from Santa Cruz Biotechnology, Inc.).

Immunofluorescence

The immunocytochemistry was done essentially as described in Engström and Rozell (1988). In brief, mbn-2 and SL2 were grown as monolayers

on glass coverslips at a density of 0.25×10^6 cells/ml. Transfected cells were diluted and spread onto the cover glasses 1 day after the transfection and left for additional 24 h. The cells were rinsed in serum-free medium, fixed in 3.7% (w/v) freshly prepared paraformaldehyde in PBS, pH 7.3 for 30 min at 37°C, rinsed in PBS 3×20 min and permeabilized with 0.2% Triton X-100 in PBS for 2 min followed by several changes of PBS. All antibody dilutions and rinsing steps were done with PBS containing 0.04% Triton X-100. The cells were preincubated with 2% normal rabbit serum for 30 min at 20°C, followed by incubation with rat anti-*Dif* antibody (1:500) (Ip *et al.*, 1993) or rat anti-dl antibody (1:500) (Rushlow *et al.*, 1989) overnight at 4°C. After rinsing, the cells were incubated with secondary FITC-labeled rabbit anti-rat antibody (1:40) (DAKO, Denmark) for 2 h at 20°C, washed and mounted in Vectashield mounting medium (Vector Laboratories, Inc.). The slides were observed in a Zeiss microscope equipped for epi-illumination and fluorescence microscopy or in a Leica confocal laser scanning microscope equipped with an argon-krypton laser.

Dif immunostaining of larval hemocytes was done by collecting the hemolymph from 3rd instar climbing larvae directly onto a tissue slide, previously coated with a thin layer of ProNectin F (Protein Polymer Technologies, Inc. San Diego), 5 µg/ml in PBS. The hemocytes were fixed and processed for staining exactly as described above.

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