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A novel association between clustered NF- κ B and C/EBP binding sites is required for immune regulation of mosquito *Defensin* genes

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Abstract

A comparative analysis identified key *cis*-acting regulatory elements responsible for the temporal control of mosquito *Defensin* gene expression. The promoters of *Anopheles gambiae* *Defensin 1* and two isoforms of *Aedes aegypti* *Defensin A* are up-regulated by immune challenge. This stimulated activity depends upon a cluster of three NF- κ B binding sites and closely associated C/EBP-like motifs, which function as a unit for optimal promoter activity. Binding of NF- κ B and C/EBP like transcription factors is confirmed by electrophoretic mobility shift assay, including supershifts with antibodies to C/EBP. κ B-like motifs are abundant within antimicrobial peptide gene promoters and most are very closely associated with putative C/EBP binding sites. This novel association between NF- κ B and C/EBP binding sites may, therefore, be of widespread significance.

Keywords

Immune regulation; *Defensin* genes; mosquito; NF- κ B; C/EBP

Introduction

Mosquitoes are among the most important vectors of human disease. *Anopheles gambiae* is a vector of malaria, while *Aedes aegypti* transmits the viral diseases yellow fever and dengue. In response to infection, mosquitoes mount an immune response (Lehane et al., 2004) which resembles mammalian innate immunity (Hoffmann & Hetru, 1992), leading to the transcription of antimicrobial peptide (AMP) genes. One group, the *Defensins*, were first fully characterized in *Drosophila* and have since been reported in both *An. gambiae* (Christophides et al., 2002; Eggleston et al., 2000; Richman et al., 1996) and *Ae. aegypti* (Chalk et al., 1995; Cho et al., 1997; Lowenberger et al., 1995).

Expression of *An. gambiae* *Defensin 1* (*AgDef1*) has been particularly well studied. In infected mosquitoes, basal levels of mRNA are strongly up-regulated in the fat body and

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Supplementary material

The following material is available for this article online: <http://www.blackwell-synergy.com/loi/imb>

Additional results and background information.

locally in the midgut and salivary gland, at times coinciding with malaria parasite penetration of the midgut or salivary gland epithelium (Dimopoulos et al., 1998).

Cis-regulatory elements involved in the expression of mammalian acute-phase proteins, particularly Rel binding motifs such as the κ B motif that binds NF- κ B, are also found in the upstream region of insect AMP genes (Hoffmann & Reichhart, 1997). Several putative κ B motifs have been identified within both *AgDef1* and *Ae. aegypti Defensin A (AaDefA)* upstream sequences (Cho et al., 1997; Eggleston et al., 2000) and the most proximal site in each promoter shown to specifically bind transcription factors in Electrophoretic Mobility Shift Assay (EMSA, Barillas-Mury et al., 1996; Shin et al., 2002). A single Rel protein, *Gambif1*, has been isolated from *An. gambiae* (Barillas-Mury et al., 1996) and shown to impact on the activity of *AgDef1* (Eggleston et al., 2000). The *An. gambiae* genome sequence also predicts an alternatively spliced isoform of *Gambif1* and a *Relish* orthologue (Christophides et al., 2002). In *Ae. aegypti* three isoforms of *Relish* (Shin et al., 2002) and a putative *Gambif* homologue (Bartholomay et al., 2004) have been identified. *Ae. aegypti Relish* knock-downs express severely decreased levels of *Defensin* following bacterial injection (Shin et al., 2003).

In mammalian systems the cooperative interaction of NF- κ B with multiple transcription factors is well documented (Siebenlist et al., 1994). Since Rel signal transduction pathways are activated in response to a range of stimuli (Perkins, 2000), control of mosquito *Defensin* expression will undoubtedly involve other transcription factor binding sites. In *Drosophila* the involvement of Rel signal transduction pathways has been demonstrated for all seven AMPs (Engstrom, 1999). However the specific binding of transcription factors to promoter sequences has only been demonstrated for the *Cecropin A1* and *Diptericin* genes. In addition to κ B motifs, *Cecropin A1* expression requires closely associated R1 and GATA factor binding sites (Kadalayil et al., 1997; Uvell & Engstrom, 2003). Two 17 base pair repeats in the *Diptericin* promoter harbour NF- κ B binding sites which act in synergy following induction (Kappler et al., 1993). The most proximal site is closely associated with an NF-IL6 or CCAAT/enhancer binding protein β (C/EBP β) binding site and an interferon consensus response element (ICRE). All three overlapping motifs are protected by DNase I footprinting (Georgel et al., 1993) and the sequence homologous to mammalian ICRE subsequently shown to enhance activity (Georgel et al., 1995). In addition to κ B-like motifs we previously identified several putative transcription factor binding sites including GATA, NF-IL6 (C/EBP β) and ICRE in the *AgDef1* promoter (Eggleston et al., 2000). However transcription factor binding to mosquito AMP gene promoters has so far only been demonstrated for NF- κ B in *Defensin* genes (Barillas-Mury et al., 1996; Shin et al., 2002).

Transgenic mosquito technology may become an important tool in the fight against transmission of insect-borne diseases. Understanding the control of gene expression following an immune challenge will yield information critical in controlling transgene expression in mosquitoes. We previously isolated an *AgDef1* genomic clone and here we isolated two *AaDefA* genes. We used a comparative approach to identify important transcription factor binding sites within these mosquito *Defensin* promoters and analysed their impact on gene expression. Here we report the characterization of a set of three NF- κ B binding sites, each closely associated with a C/EBP-like motif, which is conserved between three *Defensin* genes from two different mosquitoes. Their functional significance is demonstrated and possible models for NF- κ B/C/EBP binding discussed.

Results

Isolation and characterization of Defensin genomic clones from *Aedes aegypti*

Ae. aegypti Defensin sequences were isolated from genomic clones of the Liverpool strain. Southern blot and sequence analysis revealed two genes with similar, but not identical, coding regions (supplementary material and data not shown). One gene was very similar to *AaDefA1* (Gao et al., 1999) and the other was most similar to *AaDefA4* cDNA (Gao et al., 1999) and *DefA_{1a}* (Cho et al., 1997). We were unable to isolate genes encoding *Defensin B* or *C* isoforms previously described by Lowenberger et al. (1995). The nucleotide sequences of our *A1* and *A4* genes share 98% identity in the protein-coding region and 79% identity in the 300 bp upstream of the ATG codon. Either side of this region the sequences of the two genes are totally different. These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AY625501 (*AaDefA4*) and AY625500 (*AaDefA1*).

5' RACE analysis of the *A4* and *A1* gene products identified transcription start sites positioned 69 and 71 base pairs, respectively, upstream of the coding region and 1 and 3 base pairs, respectively, upstream of a putative arthropod initiator (consensus DCAKTY, Cherbas & Cherbas, 1993). Analysis of proximal promoter sequences (−200 to −1) identified a TATA box (TATAA) and three putative NF-κB binding sequences (insect consensus GGGRNTYYYY, Kappler et al., 1993), all highly conserved between the two genes. The κB-like motifs were described previously by Cho et al. in *DefA_{1a}* (1997). Additionally, using MATINSPECTOR software (Quandt et al., 1995), we identified C/EBP-like binding sequences (consensus TKNNGYAAK, Ryden & Beemon, 1989) closely linked with each putative κB motif and several GATA-like binding sequences (consensus WGATAR, Evans et al., 1990) located more distal to the start site (Fig. 1A and supplementary Fig. 5A). Our previous analysis of the *AgDef1* promoter also identified three κB-like motifs proximal to the transcription start site (Eggleston et al., 2000) and further investigation identified putative C/EBP binding sites in close proximity to each (Fig. 1A).

Activity of mosquito Defensin promoters in cell culture

Gene expression mediated by the upstream region of *AgDef1* (−1240 to +14) was investigated previously (Eggleston et al., 2000). In this study, promoter regions from all three *Defensin* genes, including the 5' UTR, were used *in-vitro* to drive firefly luciferase expression. Reporter plasmids were transiently transfected into either *An. gambiae* Sua 4.0 cells (Catteruccia et al., 2000) for *AgDef1*, or *Ae. aegypti* Aag2 cells (Peleg, 1968) for *AaDefA4* and *AaDefA1*. All full-length promoter constructs were significantly more active than the promoterless pGL3 vector in dual-luciferase assays and significantly up-regulated following immune stimulation with LPS (supplementary Fig. 5B).

Sets of 5' nested deletions were constructed to investigate the importance of putative transcription factor binding sites within the promoters. Initial experiments showed the importance of the proximal region extending back to include all three κB motifs. We found no significant differences between the LPS stimulated activities of constructs carrying these motifs and full length promoter constructs (supplementary Fig. 5B). Very similar patterns of up-regulation were obtained following the treatment of all transfections with either heat killed bacteria (*Escherichia coli* or *Micrococcus luteus*) or peptidoglycan (data not shown). In contrast, none of the promoters were up-regulated following treatment with laminarin (data not shown).

Pairs of putative NF-κB and C/EBP binding sequences, grouped into regions III, II and I based on their distance from the transcription start site, were added sequentially in 5' deletion constructs 2i, 2ii and 2 (Fig. 1B). Constructs were transfected into the conspecific

cell line and either untreated or immune stimulated with LPS. Relative luciferase activities for *AgDef1* following LPS treatment showed a gradual, but highly significant, increase in promoter activity with size (p1*AgDef1*: 0.69 ± 0.05 , p2i*AgDef1*: 1.40 ± 0.21 , p2ii*AgDef1*: 2.15 ± 0.16 , p2*AgDef1*: 4.00 ± 0.48 with $P < 0.001$ for all comparisons, Fig. 1C). In contrast, the activity of *AaDefA1* constructs remained very low until all three regions were included. Following immune stimulation with LPS, greater than 10 fold increase in activity was observed between p2ii*AaDefA1* and p2*AaDefA1* (0.82 ± 0.07 and 8.83 ± 0.95 , $P < 0.001$), compared to a 1.9 fold increase for the equivalent *AgDef1* constructs. Very similar results were obtained following immune stimulation with heat-killed *M. luteus* (data not shown).

Mutagenesis of putative NF- κ B and C/EBP binding sites

The involvement of individual κ B and C/EBP-like motifs in p2*AaDefA1* was investigated by site-directed mutagenesis (Fig. 2A). Following transfection and immune stimulation with LPS, all κ B mutants were significantly less active than the wild-type sequence (Fig. 2Bi). Compared to p2*AaDefA1* (1.7 ± 0.12) activities were reduced to 29% for I κ B mut (0.5 ± 0.05 , $P < 0.001$), 39% for II κ B mut (0.7 ± 0.09 , $P < 0.001$) and 16% for III κ B mut (0.3 ± 0.02 , $P < 0.001$), indicating a function for all 3 motifs. The additional mutant II κ B2 mut does not alter the C/EBP motif (Fig. 2A) but still reduced activity to 40% of wild-type, confirming functionality for the region II κ B site (data not shown). Immune stimulation with *M. luteus* produced similar results (data not shown). In addition we mutated the *AgDef1* region II κ B motif and observed a significant drop in stimulated full-length promoter activity to 41% of wild-type levels (data not shown). Mutagenesis of p2*AaDefA1* C/EBP binding motifs also impacted on promoter activity. Mutants I C/EBP mut and II C/EBP mut reduced LPS stimulated activity to 63% and 25% of wild-type respectively (0.96 ± 0.09 , $P < 0.001$; 0.39 ± 0.03 , $P < 0.001$, Fig. 2Bii). In contrast, mutant III C/EBP mut significantly increased LPS stimulated activity (2.02 ± 0.10 , $P < 0.001$), suggesting a negative regulatory effect for binding to the native C/EBP-like motif.

Binding of Nuclear Proteins to Regions I and II in EMSA

In EMSA, binding of nuclear proteins to the sequence II κ B + C/EBP in the *AaDefA1* promoter was strongly induced in LPS treated cell extracts (band a, lanes 8 and 14, Fig. 3A) and was competed by a κ B binding sequence from the *Drosophila Dipteracin* gene (Kappler et al., 1993; lane 12). However, a C/EBP binding sequence from the *Drosophila* fat body enhancer (Dittmer & Raikhel, 1997) did not compete with binding to the sequence (lane 17). Competition by an oligonucleotide containing a mutated κ B motif (lane 11) was considerably reduced compared to self (lane 9). However, mutation of the C/EBP-like sequence had no effect on competition (lane 10). The binding to region II was also competed by region I κ B + C/EBP (lane 16) and, when used as a probe, region I gave the same pattern of band retardation as region II (data not shown). These results suggest that the same (or related) Rel-type factor binds to regions I and II in these assays. Two retarded bands were observed with untreated cell extracts (b and c), both of which were incompletely competed by an NF- κ B binding sequence (lane 6) but not competed by an oligonucleotide containing a mutated NF- κ B binding sequence (lane 5). The transcription factors forming these weak complexes involving the κ B motif in unstimulated extracts are not known.

To establish whether the C/EBP-like motif in region II is capable of binding nuclear proteins we tested an oligonucleotide in which the κ B motif is mutated (Fig. 3B). Both untreated and LPS treated cell extracts bound specifically as a single band, d, to the sequence II κ B mut (lanes 2 & 8), indicating that translocation of transcription factors such as NF- κ B is not required. The retarded band was competed equally well by itself (lanes 3 & 9), the oligonucleotide II κ B + C/EBP (lanes 4 & 10) and region I (data not shown), suggesting that

the C/EBP-like motif is capable of nuclear protein binding in both regions I and II, even in the presence of the κ B-like motif. Mutation of the C/EBP motif in region II removed its ability to compete (lanes 5 & 11) whereas a *Drosophila* C/EBP binding sequence was a strong competitor (lanes 6 & 12). Due to the plasticity of the C/EBP binding sequence, unrelated sequences used as non-competitors in this study (lanes 7 & 13) contained a 7/9 match to the consensus. Therefore the unrelated sequence appears to be partially capable of competing for binding to oligonucleotide II κ B mut. We think that an abundance of Rel-type factors in the nuclear extract is masking binding to the C/EBP site when the oligonucleotide II κ B + C/EBP is used as a probe.

Further evidence in support of C/EBP binding to regions I and II in the *AaDefA1* promoter was provided by supershift experiments. Two polyclonal antibodies directed against *Drosophila melanogaster* C/EBP were used (Rorth & Montell, 1992). Antibody C135 is directed against the conserved C/EBP DNA binding domain and C138 against the last 12 amino acids of the C-terminus, which are usually variable among different species. We found that both antibodies recognized a protein(s) in untreated and LPS treated cell extracts that is involved in complexes on region I (data not shown) and II (Fig. 3 A and 3B). Antibody C138 caused a supershift of bands a, b and c (band e in lanes 22 and 27, Fig. 3A) and of band d (band e in lane 17, Fig. 3B) suggesting that C/EBP is present in each of these complexes. A weaker supershift was observed with antibody C135 (lanes 21 and 26, Fig. 3 A and lane 16, Fig. 3B) which also appeared to prevent the formation of bands a, b and c and reduce the formation of band d. This is probably because it recognized the DNA binding domain of C/EBP present in these complexes.

Discussion

By means of a comparative analysis, we identified three sets of NF- κ B and C/EBP binding sites, conserved in the region from -200 to the transcription start site, in three mosquito *Defensin* promoters. The two *Aedes* promoters (*AaDefA1* and *AaDefA4*) are totally different upstream of this region, suggesting the conservation of important response elements in genes that could have arisen by duplication.

Following transfection into insect cells, the *Anopheles* promoter (*AgDef1*) showed higher levels of basal activity, but the *Aedes* promoters were up-regulated more strongly (Fig. 1C), entirely consistent with differences in levels of *Defensin* reported *in-vivo* for the two insects (Lowenberger et al., 1999; Vizioli et al., 2001). In addition, transfection of *AaDefA1* into Sua 4.0 cells and *AgDef1* into Aag2 cells confirmed that variations in activities were due to different promoter sequences (data not shown). We did however, note that relative luciferase activities varied between batches of experiments. We believe this to be due to differences in the sensitivity of cells to both transfection (which can influence basal promoter activity) and immune stimulation.

Independent mutagenesis of κ B and C/EBP motifs within the *AaDefA1* promoter and the use of these sequences in EMSA suggest that all six sites are bound by transcription factors. In contrast, deletion constructs p2i*AaDefA1* and p2ii*AaDefA1* show minimal activity until region I is included in p2*AaDefA1* (Fig. 1C). We therefore propose that all three regions function together as a single unit for optimal activity. Data for *AgDef1* confirm the conservation of a functional unit since regions II and III have been implicated in promoter activity (Barillas-Mury et al., 1996 and this paper) and activity is further increased by the addition of region I. The number of NF- κ B binding sites was also shown to impact on the activity of the *Drosophila Dipteracin* promoter (Kappler et al., 1993).

Through EMSA we have shown that *AaDefA1* regions I and II bind NF- κ B/Rel-type proteins. Together with the binding of *Aedes Relish* to the region III sequence (Shin et al.,

2002), Rel protein binding to all three κ B motifs is confirmed. Competition using a *Drosophila* C/EBP binding sequence to a region II probe could only be observed when the κ B site was mutated. The resulting complex could be competed equally well by self or wild-type region I or II competitors, indicating that C/EBP-like sequences in both regions are able to bind nuclear extracts, even in the presence of the κ B motif. The binding of C/EBP to motifs within regions I and II was confirmed using *D. melanogaster* C/EBP antibodies in supershift experiments. Since C/EBP supershifts were evident using wild-type region I and II probes, both shown by competition assays to include NF- κ B-like proteins, we conclude that regions I and II are able to form complexes on overlapping sites that include both transcription factors.

Pairs of binding sites within our mosquito *Defensin* promoters fall into two types, with NF- κ B and C/EBP recognition sites overlapping by 4 or 5 nucleotides (*AaDefA1* regions I, II; *AgDefI* region III) or separated by 3–8 nucleotides (*AaDefA1* region III; *AgDefI* regions I, II, Fig. 1A). Mutagenesis of individual *AaDefA1* C/EBP motifs identified a role in up-regulation for those that overlap NF- κ B binding sites and down-regulation following LPS stimulation for region III, in which the binding sites are separate. In *AgDefI* these pairs of motifs are in different locations and regions II and III contribute more to stimulated promoter activity in the absence of region I (Fig. 1C). We propose that differences in numbers of overlapping vs. separate pairs of binding sites, plus their relative distances from the transcription start site, could be responsible for differences in promoter activities. In fact, the antagonistic effect observed for the *AaDefA1* region III C/EBP motif could depend on its location. In a similar situation (Prösch et al., 2001) proposed that C/EBP binding at –90 to –83 in the HCMV IE1/2 promoter, 3 base pairs downstream of a κ B motif, could inhibit an interaction between bound NF- κ B and the basal complex due to its location between them.

κ B binding motifs are conserved in *Drosophila* AMP gene promoters (Engstrom, 1999) and GATA factor binding sites have been proposed to modulate the NF- κ B response (Kadalayil et al., 1997). More recently temporal and spatial control has been confirmed for GATA factor binding on the *Drosophila Cecropin A1* promoter (Petersen et al., 1999). In contrast, GATA factor motifs are located more distal to the NF- κ B binding sites in mosquito *Defensin* genes, outside the region responsible for the majority of stimulated promoter activity in insect cells. In these genes we identified closely associated and overlapping C/EBP binding sites which we propose modulate the NF- κ B response. Members of the NF- κ B family of transcription factors are known to mediate the transcriptional induction of numerous genes in mammalian immune and inflammatory reactions and C/EBP binding motifs have also been identified in a number of these (Betts et al., 1993; Diamond et al., 2000). However, with a few exceptions which did not involve multiple elements (Brasier et al., 1990; Stein & Baldwin, 1993), those identified are not generally overlapping or adjacent to NF- κ B binding sites. We then questioned whether similar close associations of multiple NF- κ B and C/EBP binding sites could regulate immune gene expression more widely. To answer this we investigated other AMP genes in the following way. First, we note that our mutagenesis studies identify a degree of plasticity in the C/EBP consensus, with functional sites having two mismatches but only one at nucleotides 1, 5, 7 or 8, shown to exhibit the highest conservation (MATINSPECTOR). Using this degree of plasticity, we looked for putative C/EBP binding sites in 37 AMP gene promoters from nine insect species. All the sequences contained putative C/EBP binding sites within 20 base pairs of κ B motifs (data not shown). In two thirds of these promoters, putative C/EBP binding sites overlap κ B motifs by 3–7 nucleotides, compared to 5 in the functional sites within the *AaDefA1* promoter (supplementary Table 1). We therefore propose that a close association between NF- κ B and C/EBP binding sites could be important in the expression of the majority of insect antimicrobial genes. In the published literature the synergistic enhancement by Rel and C/

EBP proteins *via* an intron sequence in the *Hemolin* gene of *Hyalophora cecropia* was reported, although the C/EBP binding site was not identified (Roxstrom-Lindquist et al., 2002). Within the limited number of vertebrate AMP gene promoters studied, putative binding sites for both NF- κ B and C/EBP have also been identified (Miele et al., 1998; Pestonjamas et al., 2001; Scocchi et al., 1997; Shin et al., 2004). Adopting the same search criteria as above we identified additional C/EBP motifs and observed that half the κ B motifs within vertebrate AMP gene promoters overlapped putative C/EBP motifs (data not shown) suggesting the possibility of a similar regulatory mechanism in vertebrates.

The control of gene expression by both NF- κ B/Rel proteins and the C/EBP group of b-zip proteins has been reported in vertebrates and appears to be complex. Rel proteins dimerize and bind DNA *via* a conserved Rel homology domain. The C/EBP group of transcription factors form dimers *via* their conserved b-zip domain, causing conformation changes that allow DNA binding. Stein & Baldwin (1993) demonstrated an interaction between closely opposed NF- κ B and C/EBP in the control of Interleukin-8 gene expression and suggested independent binding of NF- κ B or C/EBP to adjacent sites and co-operative binding of both to DNA. However these two groups of proteins could also impact on promoter activity through a single recognition site and the two transcription factors acted synergistically through a C/EBP binding site but antagonistically through an NF- κ B binding site (Stein et al., 1993). Correct regulation of IL-8 expression was proposed to depend on the ratio of cellular C/EBP and NF- κ B. In addition NF- κ B/Rel proteins were shown to form interfamily heteromers *in vitro* (LeClair et al., 1992) and more recently *in vivo* (Adams et al., 2000). It is not known if such heteromers bind DNA *via* adjacent, overlapping or novel sites, or whether only one of the subunits binds DNA. Overlapping binding sites for NF- κ B and C/EBP have been identified in an acute-phase response element in the angiotensinogen promoter (Brasier et al., 1990). Binding of the two proteins was reported to be mutually exclusive and likely to depend on the relative abundance of each in *HepG2* cells. In contrast to our observations, transfection of promoter sequences with C/EBP site mutations in overlapping motifs increased both basal and IL-1 α induced activity. Stein et al. (1993) reported interactions between several different Rel and C/EBP family members, further supporting the hypothesis that interactions between these two groups of transcription factors are of relevance in the regulation of immune genes. We can therefore speculate that mosquito NF- κ B and C/EBP proteins might be able to interact in a variety of ways, possibly dependant on their relative abundances.

In conclusion, we identified a conserved set of three κ B/C/EBP-like motifs in mosquito *Defensin* promoters. All six motifs were shown to impact on promoter activity and those in Regions I and II bound NF- κ B and C/EBP transcription factors. The binding of transcription factors to Region III was not tested here but NF- κ B binding to Region III of AgDef1 and AaDefA has been demonstrated elsewhere (Barillas-Mury et al., 1996; Shin et al., 2002). We present evidence which indicates that this closely associated cluster of NF- κ B and C/EBP binding sites functions as a novel promoter module. We identified functional C/EBP binding sites by allowing some deviation from the consensus sequence, it will be of interest to see whether a similar approach could identify sets of motifs in other insect AMP or mammalian acute-phase response genes. Gene expression from such promoters could potentially involve different combinations of interactions between homo- hetero- and interfamily dimers.

Experimental procedures

5' RACE

Rapid Amplification of cDNA Ends was carried out on total RNA (4.2 μ g) isolated from 6 adult female *Ae. aegypti*, 22 h post inoculation with a needle dipped in bacteria (*E. coli* K12

RM148 and *M. luteus*, 2×10^9 /ml). GeneRacer kit (Invitrogen Ltd, Paisley, UK) was used according to manufacturers instructions with nested primers (5'-CCGGTTCCTGTGGGTAGGCACTAGTAAT-3' and 5'-GGTTTCCTCCGGCAGTTCATCGAAAAGA-3'). 5' RACE products were cloned into pCR4-TOPO (Invitrogen) and sequenced.

Construction of reporter and expression plasmids

Promoter regions for constructs p*AgDefI*-1241/+100, p*AaDefA4*-1047/+68 and p*AaDefA1*-915/+70 were amplified using forward and reverse primers that introduced 5'-*SacI* and 3' *HindIII* restriction sites for *AgDefI* or 5' *MluI* and 3' *BglII* restriction sites for *AaDefA4* and *AaDefA1*. PCR products, cloned into pCR2.1 TOPO (Invitrogen), were sequenced prior to transfer into the luciferase expression vector pGL3-Basic (Promega, Southampton, UK). Sets of nested 5' deletions were generated in the same way for *AgDefI* (2-245/+100; 2ii -171/+100; 2i -88/+100; 1-76/+100) and *AaDefA1* (2-235/+70; 2ii -179/+70; 2i -99/+70 and 1-55/+70). The Actin 5C-*Renilla* transfection control plasmid contains the *Drosophila Actin* 5C promoter (Pinkerton et al., 2000) in pRL-null (Promega).

Site-directed mutagenesis

QuikChangeII Site-Directed Mutagenesis (Stratagene, Amsterdam, Netherlands) was used to mutate sites within the p2*AaDefA1* TOPO clone. 20 ng template DNA was used with 125 ng of each HPLC purified primer (Invitrogen). Mutated products were reintroduced into pGL3-Basic following sequence analysis. Mutagenesis of κ B motifs replaced nucleotides 2-4 or 7-9 (depending on orientation) with ATT or AAT (Kappler et al., 1993). Primers 5'-ATAGTCCCTTTTGCAGATGTAAATAATCCAATTCTTAAG-3' and 5'-GCACTTTACGCTTTCGGTGATAATCACGGTAGCGTACGGTGG-3' together with their complementary reverse primers were used to generate I κ B mut and III κ B mut, respectively. For II κ B mut, where the sequence matches the consensus equally well in either direction, nucleotides 2-4 and 7-9 were changed. Primer 5'-CTTAAGGATTGTCGCGGTGATAATCCAATCATCCCGTCGCTAATG-3', followed by primer 5'-CCAATTCTTAAGGATTGTCGCGATTATAATCCAATCATCCCGTC-3', were used with their complementary reverse primers in subsequent rounds of mutagenesis. II κ B2 mut, used primer 5'-GACGGGATGATTGGTGGATCGGGGCGACAATCCTTAAGAATTG-3' and its reverse complement to mutate nucleotides 1-3 (GGT) to CCC. Mutagenesis of C/EBP motifs replaced nucleotides 6-8 (Sourmeli et al., 2003), with AGG. Forward primers: I C/EBP mut 5'-CAGATGTAAATCCCCAGGTTCTTAAGGATTGTCGCGGTGATC-3'; II C/EBP mut 5'-GATTGTCGCGGTGATCCACAGGTCATCCCGTCGCTAATG-3'; III C/EBP mut 5'-GTGATTTACACGGTAGCGAGGGGTGGCAGCTTATTCATTG-3', were all used with complementary reverse primers.

Cell culture and transfection

The *An. gambiae* Sua 4.0 cell line, established from neonatal larvae (Catteruccia et al., 2000) and the *Ae. aegypti* Aag2 cell line, which originated from embryonic tissues (Peleg, 1968), were maintained at 27 °C in Schneider's medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin (Sigma-Aldrich, Poole, UK). Transfection was mediated by FuGENE 6 (Roche, Diagnostic Ltd., Lewes, UK) following the manufacturers instructions. Briefly, 5×10^5 cells were plated in 500 µl medium in individual wells of 24-well plates and grown to 60-70% confluence. Cells were transfected using 6 µl FuGENE per 4 wells together with 4 µg DNA (Sua 4.0) or 2 µg DNA (Aag2). Actin 5C-*Renilla* transfection control plasmid was adjusted for sets of experiments (10-100 ng) such that *Renilla* activities were not less than 1% of firefly luciferase activities. Immune

challenge, 24 h post transfection, was 100 µg/ml lipopolysaccharide (LPS, *Escherichia coli* serotype 026:B6, 15×10^6 EU/mg, Sigma-Aldrich).

Dual-luciferase reporter assays

Firefly and *Renilla* luciferase activities were measured using the Dual-Glo system (Promega). Briefly, cells were lysed in 24 well plates with 75 µl PBS and 75 µl luciferase reagent and transferred to a white 96 well plate to measure firefly luciferase activity. *Renilla* activity was measured after 10 min incubation with 75 µl Stop & Glo reagent. Following normalization to *Renilla* activity, luciferase data from 2 or 3 experiments, with 4 replicates in each, were pooled for analysis. Log data for each set of experiments (separated by treatment when the entire data failed to fit a normal distribution) were analysed by ANOVA (General Linear Model) with Tukey's pair-wise comparisons. For each set of experiments, all constructs within a given treatment were compared to all other constructs. Very highly significant differences are indicated by different letters for untreated samples and letters with an apostrophe for LPS treated samples.

Electrophoretic mobility shift assays

Crude nuclear extracts were prepared from 1×10^8 Aag2 cells using a modified version of the method described by Staal et al. (1990). Nonidet P-40 was substituted for IGEPAL CA-630 (Sigma) and buffer C was 20 mM HEPES pH 7.9/420 mM NaCl/1.2 mM MgCl₂/0.2 mM EDTA/10% glycerol/0.5 mM dithiothreitol/0.5 mM phenylmethylsulphonylfluoride supplemented with 5 µg/ml each of pepstatin, leupeptin and aprotinin (Sigma-Aldrich). Aag2 cells were treated with 100 mg/ml lipopolysaccharide (LPS) for 16 h prior to extraction for LPS treated extracts. Oligonucleotides corresponding to different regions of the *AaDefA1* promoter were cloning grade HPLC purified (Invitrogen). EMSAs were performed as described in Promega Technical Bulletin 110. Competitor oligonucleotides were added to a 200-fold excess. Antibodies C135 and C138 are directed against *D. melanogaster* C/EBP (Rorth & Montell, 1992), C135 binds the conserved C/EBP DNA binding domain and C138 binds the last 12 amino acids of the C-terminus. For supershifts, 1 µl of antibody was added to nuclear extracts and incubated for 20 min on ice prior to addition of labelled oligonucleotide.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Sequence and activity of transcription factor binding regions. (A) Alignment of putative C/EBP (dashed open boxes, consensus TKNNGYAAK) and insect κ B (open boxes, consensus GGGRNTYYYY) motifs, identified within the proximal promoters of *AgDef1* and *AaDefA1*. Arrowheads indicate orientation and lower case are bases that do not match the consensus. Pairs of binding sites are grouped into regions I, II and III based on their distance from the transcription start site, the nucleotide numbers of which are indicated. (B) Promoter deletions. The upper section indicates the relative location of C/EBP-like motifs (filled bars) and κ B-like motifs (open bars) in the *AgDef1* and *AaDefA1* promoters. The positions of regions I, II and III relative to the transcription start site (arrow) are indicated. The bottom

section indicates the relative sizes of 5' deletion constructs, designed to sequentially add regions III, II and I, cloned upstream of a firefly luciferase reporter (open arrow). Nucleotide numbers are p*AgDefI*: -245/+100, -171/+100, -88/+100, -76/+100 and p*AaDefAI*: -235/+70, -179 +70, -99/+70, -55/+70 for constructs 2, 2ii, 2i and 1, respectively. (C) Normalized luciferase activities (relative light units, RLU; *Y*-axis) of deletion constructs following transfection into Sua 4.0 cells (*AgDefI*) or Aag2 cells (*AaDefAI*). Transfections were untreated (open bars) or immune stimulated with 100 µg/ml LPS (filled bars). The means and standard errors of 12 replicates, performed on 3 separate occasions are shown. Very highly significant differences between constructs within a treatment ($P < 0.001$) are indicated by a or b for untreated cells and a', b', c' or d' for LPS treated cells.

Figure 2.

Mutagenesis of *p2AaDefA1*. (A) Nucleotide sequences of promoter regions I, II and III. Putative transcription factor binding sites are indicated by horizontal open boxes. Nucleotide changes introduced in each mutant are shown in vertical boxes. (B) Normalized luciferase activity (relative light units, RLU; *Y*-axis) following transfection into Aag2 cells of individual (i) NF- κ B or (ii) C/EBP binding site mutants. Transfections are either untreated (open bars) or immune stimulated with 100 μ g/ml LPS (filled bars). Means and standard errors of a minimum of 8 replicates, performed on at least 2 separate occasions are shown. Very highly significant differences between constructs within a treatment ($P < 0.001$) are indicated by a or b for untreated cells and a', b', c' or d' for LPS treated cells.

Figure 3.

AaDefA1 Electrophoretic Mobility Shift Assays. Binding of crude nuclear extract (20 μ g) from untreated ('untreated') and LPS treated ('LPS', 100 mg/ml, 16 h) Aag2 cells to (A) oligonucleotide II κ B + C/EBP (5'-TCGCGGTGATCCACCAATCAT-3') and (B) oligonucleotide II κ B mut. (5'-TCGCCCCGATCCACCAATCAT-3'). Competitors were non-e (-), II C/EBP mut. (5'-TCGCGGTGATCCACAGGTCAT-3'), κ B *D. mel.* (5'-TGACATCGGGGATTTCCTTTTGGAAA-3', 21), an 'unrelated' sequence (5'-AAAAAGCGTTTTTCGCTCGTACGTT-3'), I κ B + C/EBP (5'-AGATGTAAATCCCCCAATTCT-3') and C/EBP *D. mel.* (5'-TACAATGTTGCAATCAGCGG-3', 29). The consensus-like sequences are shown in bold and the mutated nucleotides are underlined. Complexes formed with probe II κ B + C/EBP were 'a' with LPS treated extracts or 'b' and 'c' with untreated extracts. Probe II κ B mut. formed complex 'd' with either extract. Antibodies C135 and C138 are directed against *D. melanogaster* C/EBP (Rorth & Montell, 1992) and generate supershift complex 'e' with either probe.