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## **Immune stimulation of *Anopheles gambiae* 4a3B cells induces chromatin reorganization at the Defensin 1 gene**

**Sandra Rosas-Madrigal, Alejandra Cabrera, Verónica Valverde-Garduno**

### **Abstract**

Mosquito innate immunity is a critical component in the transmission of *Plasmodium* parasites that cause malaria in human populations. Molecular mechanisms involved in regulation of transcription of mosquito innate immunity genes are not yet fully understood. Here, the chromatin conformation at the *Defensin 1* (*Def1*) gene was investigated in the *Anopheles gambiae* derived, immune-responsive, 4a3B cell line. Our aim was to characterize chromatin structure, before and after immune challenge, at this innate immunity gene. Here we report that, upon immune stimulation with a heat-killed bacterial challenge, *Def1* promoter sequences become co-localized with an open chromatin site. This indicates that the *Def1* promoter undergoes a dynamic chromatin transition upon immune challenge. In contrast, promoter-flanking sequences and exon sequences were not nucleosome depleted in the same samples, obtained from immune stimulated cell cultures. Taken together, these results suggest participation of chromatin modification in the transcriptional activation of the *Def1* gene.

**Keywords:** *Anopheles gambiae*; innate immunity, *cis* regulatory element; open chromatin; promoter, immunity gene.

### **1. Introduction**

Mosquito innate immunity is a critical component of vectorial competence for the transmission of *Plasmodium* parasites. This relevance has led to intense research to characterize mosquito innate immunity system components and function. Innate immunity effectors are directly coded on the mosquito genome. As a consequence, the main regulatory mechanism for many immunity effector genes is through regulation of transcription. Key factors involved in transcriptional regulation of mosquito immunity effectors have been identified [1]. However, little is known regarding the mechanisms through which these factors activate transcription, beyond their DNA binding activity. Here we provide evidence for the potential participation of chromatin reorganisation during transcriptional activation of an *Anopheles gambiae* immunity gene. Defensin 1 is an effector molecule that contributes to the robust innate immune response mounted by *An. gambiae* when confronted by invading microorganisms [2]. The corresponding *Defensin1* (*Def1*) gene has been useful as a model to study immunity gene transcriptional regulation. *Def1* is also expressed in the haemocyte-like, *An. gambiae* derived, 4a3B cell line, where its transcription is also inducible by immune challenge [3]. Previous in vitro functional studies, in Sua4 cells, have demonstrated the promoter activity of a proximal upstream sequence at the *Def1* gene locus in *An. gambiae* and upregulation upon immune challenge [1]. As all genomic sequences, *cis*-regulatory regions carry out their functions in the context of chromatin. The structure of this hierarchially organized nucleoprotein complex is highly correlated with the function of underlying sequences [4]. *Cis*-regulatory regions contain specific protein complexes that displace or destabilize nucleosomes making their underlying sequences more accessible [5,6]. This makes active *cis*-regulatory sequences highly susceptible to nuclease digestion and lead to the establishment of DNaseI sensitivity assay to identify them [7]. This method has been combined with detection by qPCR for defined individual loci [8]. DNaseI sensitivity assays have also been coupled with microarrays and next generation sequencing for genome wide identification of regulatory sequences [9,10]. More recently an additional, efficient method for open chromatin profiling was developed from serendipitous findings [11]. It was discovered during the course of chromatin studies of the yeast *Saccharomyces cerevisiae*. This method was then applied to human cells and described later as Formaldehyde Assisted Identification of Regulatory Elements [12].

It has also been applied successfully to other mammalian cells and tissues, generating genome wide tracks of *cis*-regulatory sites [13]. Here we applied this recently developed method to investigate the chromatin conformation at the *Defl* locus in immuno stimulated 4a3B cells. Gene regulation through chromatin rearrangements is a well known mechanism through which eukaryotic cells can maintain a tight control over the process of transcription [14,15]. Activation mechanisms of stimulus responsive genes can also involve chromatin remodeling [16]. *An. gambiae* *Defl* gene is upregulated, along with other innate immunity genes, when 4a3B cells are challenged with heat-killed bacteria [3]. In order to investigate whether chromatin remodeling could be involved in transcriptional activation of this gene, chromatin accessibility was analysed in nonstimulated and immuno-stimulated 4a3B cell cultures. Evidence was found indicating that upregulation of the *Defl* gene increases accessibility of promoter sequences in immuno stimulated cell cultures. This suggests that chromatin remodeling and chromatin remodeling factors may participate in transcriptional regulation of innate immunity genes in *An. gambiae*. In addition, to facilitate the study of *cis* regulatory sequences in this mosquito cell line, a rapid and low cost open chromatin profiling protocol was generated. This rapid, small-scale protocol for open chromatin profiling successfully discriminate a *cis*-regulatory site from non-open chromatin in 4a3B cells.

## 2. Materials and Methods

**2.1 Cell culture and bacterial challenge:** *An. gambiae* derived 4a3B cells (MRA-919) were grown in 10% FBS supplemented *Drosophila* Schneider medium and incubated at 27°C, as has been previously described. For detection of nucleosome depleted regions with the FAIRE method two 75cm<sup>2</sup> bottles 90% confluent of 4a3B cells were used. For the detection of nucleosome depleted regions with the fast prep method one 25cm<sup>2</sup> bottle of 4a3B cells 90% confluent was used. A 1:1 mixture of heat killed *Micrococcus luteus* (ATCC 9648) and *Escherichia coli* (ATCC 25922) was used to challenge cell cultures with 1000 bacteria/cell, for 4 hours.

**2.2 Chromatin structure characterisation:** Open chromatin profiling was carried out by the formaldehyde assisted identification of regulatory elements method [17], followed by qPCR. A newly modified, rapid and low cost, open chromatin protocol was also applied to detect open chromatin by standard PCR and gel staining. For this fast protocol cells were fixed by adding 1% formaldehyde in Schneider medium and incubation at room temperature for 5 minutes on an orbital shaker at 80rpm. Fixation was quenched by adding 2.5M Glycine to a final concentration of 125mM and incubation at room temperature for 5 minutes on an orbital shaker at 80rpm. Fixed cells were harvested by centrifugation at 1,500g for 5 minutes at 4°C. Cells were then washed three times with cold PBS supplemented with PMSF and supernatant was discarded after the last wash (note: at this step fixed cells can be snap frozen with liquid nitrogen or ethanol-dry ice, and stored at -70°C for several weeks). Pellets of 10<sup>8</sup> fixed cells were thawed and lysed in 10 ml lysis buffer containing 150mM NaCl, 50mM Tris-HCl pH 7.5, 5mM EDTA, 0.05% NP40 (IGEPAL) and protease inhibitors cocktail (SIGMA P8340) for 15 minutes at 4°C, with gentle rocking. Lysate was spun at 1,000 g for 5 min at 4°C and the supernatant was discarded. To resuspend pellet, 1 ml lysis buffer, containing 1% SDS, was added per 5x10<sup>8</sup> cells, prior to sonication. Chromatin shearing was carried out

in 1.7 ml micro centrifuge tubes filled with 1 ml sample and then thirty cycles of pulses of sonication for one minute followed by 1 minute incubations on ice were applied (monitoring chromatin fragmentation: a small aliquot can be used to monitor the extent of chromatin fragmentation). Once the chromatin was sheared (so that the majority of fragments range between 200 and 700 base pairs), samples were extracted twice with phenol-chloroform. Supernatant was incubated with RNase A for 30 minutes at 37 °C and then extracted with phenol-chloroform. A final extraction of the aqueous phase with chlorophorm (chlorophorm: isoamyl alcohol 24:1) was applied. To precipitate DNA 1/10 volume of 3M NaAc (pH 5.2) and 1µl of glycogen 20mg/ml were added and mixed by inverting the tube gently but vigorously. 2.5 volumes of 96% ethanol were then added and mixed thoroughly by tube inversion. Mixed samples were then incubated at -20 °C overnight for a thorough precipitation. To pellet DNA, samples were spun at 14,000 rpm in a micro centrifuge for 30 minutes at 4 °C. DNA pellets were washed with 70% ethanol and the DNA resuspended in 25 µl 1x TE. Once extracted, DNA can be further purified with columns (ZYMO Research Clean-up and concentration 5 columns were used in this case). For low cost detection of proximal active *cis*-regulatory sequences we applied a semi quantitative approach by standard PCR. 10 ng DNA from open chromatin sample were used per 50µl PCR reaction. 5 µl PCR reaction aliquots were extracted every 5 cycles from cycle 20 to 35 to compare relative quantities of open chromatin DNA fragment products, relative to control sample. Gel band fluorescence intensities were measured with the ImageJ [18] software after ethidium bromide staining.

**2.3 qPCR:** Real Time PCR reactions were carried out with the SYBR Advantage kit from Clontech following the manufacturer instructions on a FAST 7500 Thermal Cycler from Applied Biosystems. All oligonucleotide pairs were validated by melting curve analysis and dynamic range (SYBR Green qPCR assays). Fold enrichment was calculated by the Delta Delta Ct method, relative to the control sample, using 1ng sample DNA as template. Oligonucleotide pairs directed to the proximal upstream region and one amplicon directed to the coding region of the *Defl* gene were designed for enrichment quantitation of target sequences in open chromatin preparations. Oligonucleotides were designed using the CLC Main Workbench software.

**2.4 PCR reactions and gel band quantitation:** Fluorescence of gel bands from PCR products were analysed on Ethidium Bromide stained gels using the ImageJ software [18]. Fold increase (or decrease) in the open chromatin enriched samples was determined by using the control aliquot PCR products as reference, for each amplicon.

## 3. Results and Discussion

First, oligonucleotide pairs were designed to analyse chromatin structure at the *Defl* proximal region by FAIRE-qPCR. Sequences from the *Defl* promoter and flanking non-regulatory sequences were used as targets to design the PCR assay. These included an amplicon overlapping *Defl* second exon and part of the intron. All sequences were retrieved from the VectorBase *An. gambiae* genomic sequence database [19]. To test these amplicons, total DNA extracted from 4a3B cell cultures was used as template. Oligonucleotides were tested to verify the length of PCR products by gel electrophoresis. All

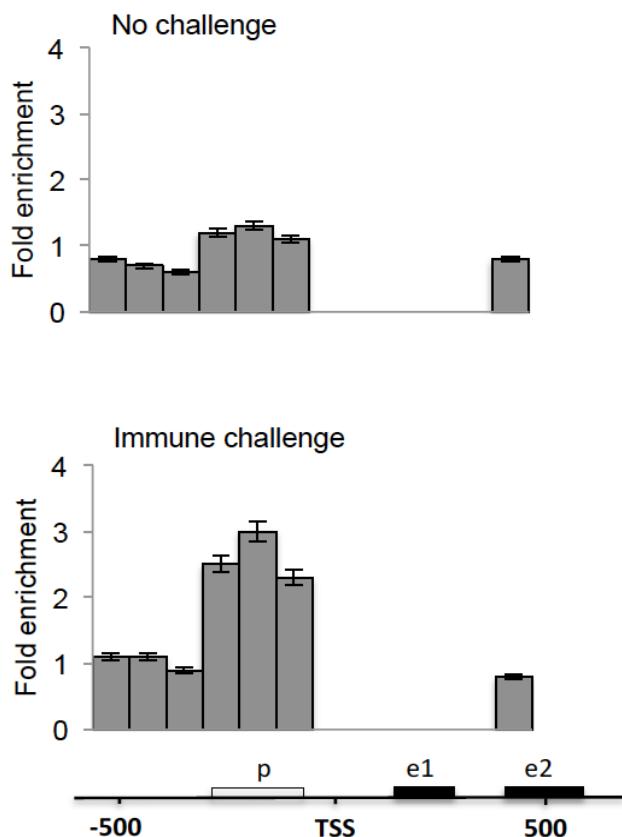
amplicons on Table 1 were shown to have the expected length and were therefore further characterized and validated for the accessibility assays. These oligonucleotide pairs were

subsequently used in Real Time PCR assays (SYBR-Green based) for relative quantitation of DNA sequences in open chromatin and control samples.

**Table 1:** Oligonucleotide pairs used for PCR and qPCR.

Amplicon	Forward primer (5' to 3')	Reverse primer (5' to 3')
1	GTACTTACCGAGCTCCCTT	ATATGCATCGAGACTGCTACT
2	TACTTACCGAGCTCCC	GAACGTACGAAGGCTCG
3	CGTTGAGTCAAACGTTAGT	GGGAAGCTCGGTAAAGTA
4	GAAGGACAACTAGGAAGGACA	AACGTTGACTCAACGGAGAC
5	ATTGAAGCAAATGCACACCTG	TGTTTGCTCCTCCTAGTTGTC
6	GCTTGCAACGTTCGAAAAGT	GGGCAGGTGTGCATTGCT
e2	ATTAGTGGACGAACGTCCCAGG	ATACAGTGAGCGGCACAAAGGC

Next, using the set of amplicons described and tested as above, the chromatin structure at the *Defl* locus was investigated. The 4a3B cell line has been previously stimulated by immune challenge resulting in upregulation of transcription of multiple innate immunity genes and therefore described as immune-responsive [3]. It has been shown that the *Defl* gene is expressed by 4a3B cells and its transcription is also upregulated upon stimulation by a bacterial immune challenge. Here 4a3B cell cultures were stimulated by a mix of Gram-positive and Gram-negative heat-killed bacteria (see materials and methods). After four hour stimulation, open chromatin fractions were obtained from these cell cultures. Relative enrichment of sequences in the open chromatin fraction was then calculated relative to a control sample.

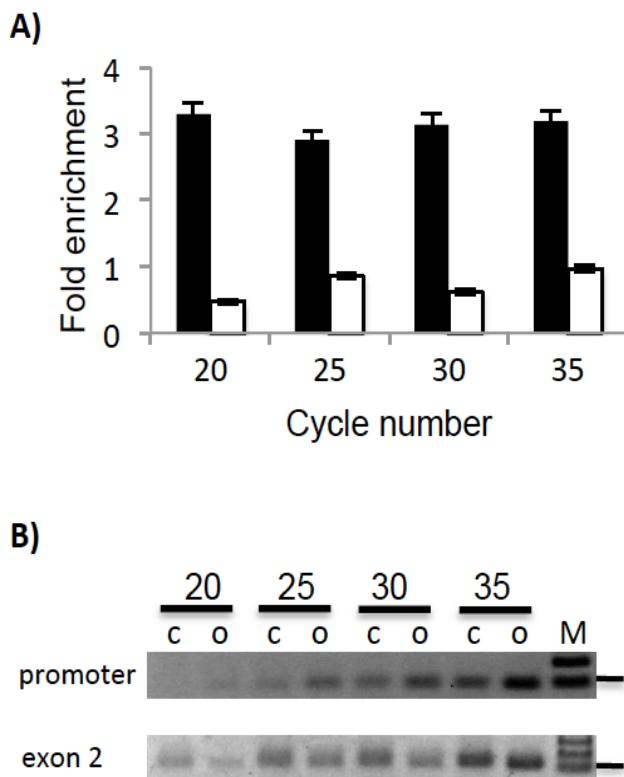


**Fig 1:** The *Defl* promoter lies within an open chromatin site in immune stimulated 4a3B cells. Open chromatin profiling for the proximal *Defl* locus. Grey bars indicate enrichment for each amplicon (1 to 6 and e2, see Table 1) in the open chromatin sample relative to a control sample. Bottom: diagram of the *Defl* locus, coordinates are in base pairs relative to the transcription start site (TSS). The open box indicates the position of the promoter and black boxes indicate exons.

Figure 1 show that sequences detected by amplicons overlapping the *Defl* gene promoter (amplicons 1 to 3) are enriched in immuno stimulated, open chromatin enriched, samples. Highest enrichment was up to 3 fold, for the amplicon that overlaps sequences around the promoter central region. Amplicons flanking the promoter, at its 5 prime end, are less enriched or non-enriched. This is also the case for the PCR amplicon overlapping sequences for the intron and second exon, approximately 500 bp downstream from the transcriptional start site (TSS). In contrast, there is very little enrichment of promoter sequences (a maximum of 1.3 fold) in samples derived from noshimulated cell cultures (Fig 1 Top graph). These results indicate that the promoter is in an open chromatin conformation in immune stimulated 4a3B cells. These data suggest that *Defl* promoter could be activated by changes in chromatin conformation upon immune challenge in these cells. The amplicon overlapping exon sequences does not become enriched, indicating that accessibility of sequences at this region is not modified by immune stimulation in 4a3B cells. This may imply that *Defl* coding sequence is in a nucleosome associated conformation in 4a3B cells, since most DNA associated with nucleosomes is not accessible or has low accessibility. In summary, these data suggest that the *Defl* promoter undergoes a dynamic chromatin conformation transition. It changes from a non-open chromatin state to the formation of an open chromatin site upon immune stimulation. These data suggest the potential participation of chromatin remodeling factors and mechanism in the transcriptional activation of *An. gambiae* innate immunity effector genes.

The FAIRE preparations described above required two 75cm<sup>2</sup> cell culture bottles of 4a3B cells. To reduce the amount of cells required, the number of steps and the cost of the assay, a fast prep alternative protocol was generated. Based on previous methods, a fast prep, low cost protocol was established for the detection of proximal open chromatin sites in 4a3B cells. Modifications were introduced and a single lysis buffer was generated after adjusting detergent concentrations for an effective cell lysis. The buffer used for the results presented in Figure 2 is as described in materials and methods section. Fragmentation by sonication is a critical step and extensive analysis and advice in carrying out this step has been reported before. For this analysis two selected PCR oligonucleotide pairs from those validated as indicated in the previous section were used. One PCR amplicon overlapping the promoter sequence and the amplicon overlapping the coding region were selected to assess the rapid protocol. The small-scale fast prep protocol was applied to immune stimulated cell cultures from one 25cm<sup>2</sup> bottle at 90% confluence for each open chromatin preparation. The promoter

PCR amplicon shows three-fold enrichment, in the fast prep samples relative to control DNA (Fig 2). This amplicon is detectable from PCR cycle 20 and enrichment is detected through cycle 35. In contrast, the coding region PCR amplicon becomes depleted at PCR cycle 20. These results show that the rapid, low cost method is able to discriminate nucleosome-depleted regions.

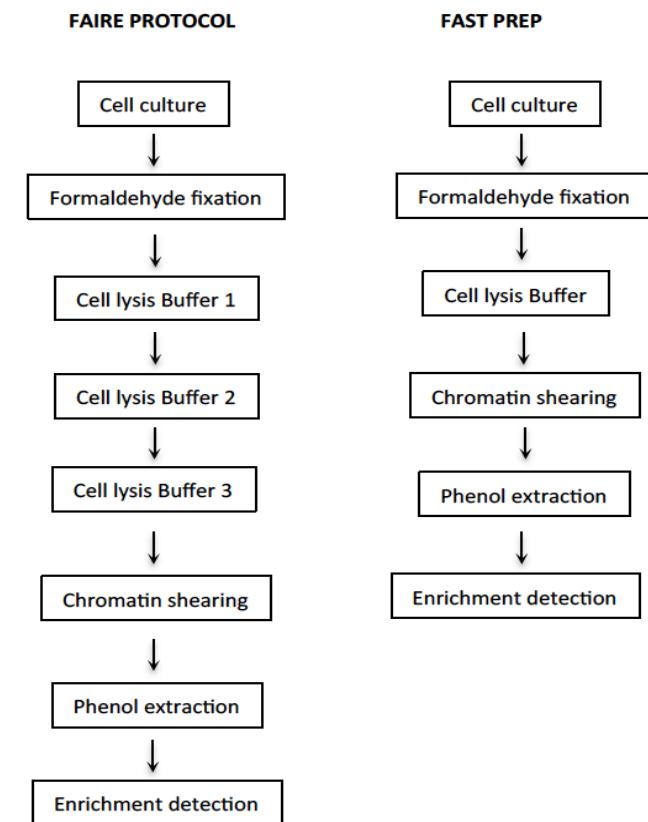


**Fig 2:** Open chromatin is detected by a fast prep protocol at the *Defl* promoter by semiquantitative PCR. Fast prep open chromatin sample and control aliquots were used as templates for semi quantitative PCR detection. Aliquots from the PCR reaction were taken at cycles 20, 25, 30 and 35 for each amplicon. Fluorescence intensities of PCR products were measured on Ethidium Bromide stained gels with the ImageJ software. A) The fold increase or decrease was determined by the ratio of fast prep open chromatin sample relative to control sample. Black bars correspond to an amplicon located at the promoter region and white bars correspond to an amplicon located at the *Defl* second exon. B) PCR product fragments were migrated in agarose gels and stained with ethidium bromide. Numbers indicate cycle number; c, control sample; o, open chromatin sample; M, molecular weight marker; horizontal line beside gel images indicate the 100 bp marker. Top: promoter amplicon. Bottom: exon 2 amplicon.

A comparison of key steps between the two methods applied in this work is shown in Figure 3. The fast prep method, by reducing steps, results in a minimal loss of sample, requiring smaller cell cultures and a shorter time for execution of the technique. We suggest that application of this low cost protocol can be useful to narrow down the number of molecules to be tested in other functional assays. For instance it can be used to select sequences of putative *cis*-regulatory elements to test in reporter assays. In this case the number of fragments to be cloned and the number of transfection assays can be reduced. It can also be applied to narrow down the sequences to be tested for DNA-protein interaction assays (Gel shift). For this purpose DNA molecules can be synthesized preferentially for sequences lying within the open chromatin enriched PCR amplicons.

Both open chromatin preparation methods are consistent in

showing that the *Defl* promoter lies within an open chromatin site in immuno stimulated cells. Overall, these open chromatin profiling data indicate that the *Defl* promoter undergoes chromatin remodeling during transcriptional activation in 4a3B cells. Recent evidence has shown that chromatin remodeling complexes and other chromatin regulators participate in the transcriptional regulation of the innate immune response in mammals and *Drosophila* [20-22]. In particular, the *Drosophila* BAP complex (a component of the Brahma ATP-dependent chromatin remodeling complex) has been shown to be required in flies infected with Gram negative bacteria for antimicrobial peptide synthesis and survival [23]. Here we found that the same immune stimulation that upregulates de *Defl* gene also induces chromatin reorganization at the promoter. These data are consistent with the potential participation of chromatin remodeling factors in mosquito innate immunity gene regulation. Further investigations will be required to identify the factors and precise chromatin modifications that take place during transcriptional activation of this gene.



**Fig 3:** Open chromatin profiling protocol comparison. Diagram shows key steps in preparation of open chromatin samples. The main difference between protocols is the use of a single step for cell lysis in the fast prep protocol; detection can be done by qPCR or semi quantitative PCR. Other steps are carried out in the same way in both protocols.

#### 4. Conclusion

The *Defl* transcript has been previously shown to be upregulated upon immune challenge, in 4a3B cells, indicating that the promoter for this gene is activated by immune stimulation [3]. Two transcription factors, one from the NF- $\kappa$ B family (REL) and C/EBP have been shown to participate in transcriptional regulation of this gene in immuno stimulated cells [1]. However, the mechanisms of activation in which these factors participate remain unknown. Here we

show, by open chromatin profiling of *An. gambiae Defl* gene proximal regions in 4a3B cells, that the accessibility of promoter sequences is increased upon immune challenge. Results presented in this work suggest that the *Defl* promoter undergoes a chromatin transition upon immune challenge. These data suggest the potential participation of chromatin remodeling factors and mechanism in the transcriptional regulation of mosquito innate immune response.

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