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Transcriptional regulation of *CYP6M2* gene in the mosquito *Anopheles gambiae* cell line

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Abstract
Effective malaria vector management involves monitoring the insecticide susceptibility of mosquito populations. The cytochrome P450 *CYP6M2* is often expressed at higher levels in *Anopheles gambiae* mosquitoes that are resistant to permethrin than in permethrin–susceptible individuals. The mechanism by which *CYP6M2* is up-regulated in *An. gambiae* is yet to be identified. In this study, factors underpinning the expression of *CYP6M2* in resistant and susceptible strains of *An. gambiae* were investigated. The activity of the region immediately upstream of *CYP6M2*, hypothesized to contain the promoter for this gene, was compared between the insecticide-resistant Tiassalé and insecticide-susceptible Kisumu strains using a reporter assay system. Thus the pGL3-*CYP6M2*_Tias and pGL3-*CYP6M2*_Kis reporter constructs were transfected into an *Anopheles gambiae* Sua 5.1* cell line to measure luciferase activity as a surrogate of promoter activity. The pGL3-*CYP9M10*-ISOP450 promoter construct from *Culex quinquefasciatus* with known promoter activity was used as a positive control. Transfected cells were challenged with 40 µM and 10 µM concentrations of permethrin diluted in methanol for 24 hours post transfection. Luciferase reporter gene assays revealed that the putative *CYP6M2* promoter for both the resistant and susceptible strains of *An. gambiae* did not show any reporter assay activity whereas luciferase activity was observed in the pGL3-*CYP9M10*_ISOP450 from *Culex quinquefasciatus*. There was no reporter activity in the insecticide challenged cells except for the *Culex*-derived positive control suggesting a possible repressor effect of the *An. gambiae CYP6M2* putative promoter region. Further bioinformatic analysis of the 896 bp *CYP6M2* revealed the occurrence of high GC ratio content (49), a large number of putative SP1 (D-Sp1) sequences (29) and CCAT (3) tetra nucleotide repeats which may act to repress transcription. Further work is necessary to determine the regions driving expression of this gene.

Keywords: *Anopheles gambiae, CYP6M2, CYP9M10, Culex quinquefasciatus,* Permethrin.

1. Introduction
The control of disease vectors of human and veterinary importance including mosquitoes relies heavily on the use of synthetic chemical insecticides [1, 2, 3, 4]. In addition to the persistent nuisance, they also transmit a number of diseases through blood feeding causing morbidity, mortality, discomfort, and heavy economic losses [5, 6, 7]. Insecticides play a central role in the control of mosquito vectors, including *Anopheles gambiae*, the vector of malaria. However, this is increasingly threatened by the development of resistance to every chemical class of insecticides, such as organochlorines, carbamate, organophosphate pyrethroid [8, 9, 10]. Pyrethroid insecticides in particular are widely used both to treat bed nets and in indoor residual spray programs in efforts to control the transmission of malaria [11, 12, 13, 14]. Insecticide resistance to pyrethroids in *Anopheles gambiae* is a major public health concern [15, 16]. The mechanisms by which *Anopheles gambiae* acquires resistance to insecticides are primarily elevated levels of detoxifying enzymes (metabolic resistance) including cytochrome P450 monooxygenases (P450s) and target-site insensitivity [17, 18, 19]. Cytochrome P450 mono- oxygenases (P450 or CYP) constitute the largest gene superfamily of structurally diverse and functionally versatile haem-containing enzymes with more than 15,000 known genes distributed across all living organisms including insects [20, 21]. Previous microarray studies have shown that overexpression of P450s has been observed in several pyrethroid resistant insects such as increased expression of *CYP6D1* in pyrethroid resistant *Musca domestica* [22], *CYP9J28* in pyrethroid resistant *Drosophila melanogaster* [23, 24], *CYP6B7* in a pyrethroid resistant strain of *Helicoverpa armigra* [25], *CYP6E1* in *Culex pipiens quinquefasciatus* [26], *CYP6P3* in pyrethroid resistant *Anopheles gambiae* [27, 18, 19] and overexpression of *CYP6M2* in pyrethroid resistant *Anopheles gambiae* [27, 19] . The increased threat to malaria control due to insecticide resistance associated with Cytochrome P450s necessitates the need to understand the mechanisms involved in this resistance [27] and this will

enhance the provision of a more effective diagnostic monitoring of metabolic based resistance development [28, 29]. Previous studies revealed that *CYP6M2* is established to be directly involved in the acquirement of insecticide resistance in *Anopheles gambiae* [30, 11, 31, 15, 32]. Whilst *CYP6M2* has been identified as having a role in insecticide resistance, the regulatory mechanisms involved in the over-expression of this gene is yet to be identified. Our previous research identified the *Anopheles gambiae* genes AGAP005300 (*Nf2e1*)/AGAP003645 and AGAP010259/ AGAP009748 as orthologs to Cap 'n' Collar isoform C (*CnCC*) / Kelch-like ECH-associated protein 1 (*Keap* 1) and / Spineless (*ss*) / Tango (*Tgo*) known to up regulate Cytochrome P450s in *Drosophila melanogaster* [33, 34, 35]. These are also orthologs to Nuclear factor erythroid 2- related factor 2 (*Nrf2*) / Kelch-like –ECH protein 1 (*Keap* 1) and Aryl hydrocarbon nuclear translocator (*ARNT*) signaling pathways in mammals respectively [36, 37]. The role of the orthologs of these genes in cytochrome P450 regulation in *Anopheles gambiae* is yet to be studied. Previous research in other insect species addressed the mechanisms that underpin this regulation, mapping critical promoter elements that are required for P450 gene induction in response to the xenobiotic phenobarbital (PB) in *Drosophila melanogaster* [38, 39, 40, 41]. Insect cell lines have been important working models for research in applied entomology since the first cell line from a moth was developed by Grace in 1962 [42, 43, 44]. Cells from *Anopheles gambiae* (5.1* and Sua 5B), *Aedes albopictus* (C6/36 cells), *Drosophila melanogaster* (S2 cells) have also been used to investigate different aspects of cellular response to stimuli and insect immunity [45, 46, 47]. Cell lines from the malaria vector *Anopheles gambiae* display powerful humoral cellular response to xenobiotics [43]. In this study, we employed dual Luciferase reporter assays in insect cell lines of *An. gambiae* to investigate the regulation of cytochrome P450 *CYP6M2* gene expression, determine the activity of putative promoters from insecticide resistant and insecticide susceptible strains, and to investigate the induction of expression by exposure to insecticides *in vitro*. This research will provide an insight into the regulatory mechanisms involved in permethrin insecticide resistance in *Anopheles gambiae*.

2. Materials and methods

2.1 Chemicals used in this study

Table 1: Chemicals used in this study, storage conditions and manufacturers

Chemical	Stock concentration and storage	Treatment concentration	Manufacturer
Permethrin	1 mM diluted in methanol, stored at -20 °C	40 µM	FMC Corp. USA
Permethrin	1 mM diluted in methanol, stored at -20 °C	10 µ M	FMC Corp. USA
Methanol	40 µl Methanol diluted in 960 µL media, stored at -20 °C	4%	ReAgent Chem., UK

2.2 Construction of the expression vector

In order to facilitate construction of luciferase assay reporter vectors, we earlier cloned the 5' upstream region of *CYP6M2* amplified from the Tiassalé and Kisumu strains into the pJET1.2 cloning vector to produce, pJET-*CYP6M2* Tiassalé (Tias) (930 bp) and pJET-*CYP6M2*_Kisumu (Kis) (896 bp). These were digested with *Bgl*II, ligated into pGL3-Basic and sequenced [48]. PGL3-*CYP6M2*_Tias & pGL3-*CYP6M2*_Kis constructs were grown in *E. coli* (200 mL), and purified using a Qiagen midi prep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions before ethanol precipitation and re-suspension in sterile water.

2.3 *Anopheles gambiae* Sua 5.1* cell culture

The *An. gambiae* cell line Sua 5.1* cell line was established with minced neonate *Anopheles gambiae* (within an hour of hatching) using modified protocols [49, 50] and received from the Kafatos Lab, Imperial College London, UK. *Anopheles gambiae* Sua 5.1* cells were kept in continual culture at 28 °C in Schneider's Drosophila medium (Life technologies, Paisley, UK) supplemented with 10% fetal calf serum (FCS) and 100 U/ml Penicillin and 100 mg/ml Streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Approximately 5×10^6 cells were seeded on 24-well plates and cultured for 24 h before transfection and allowed to reach 60-70% confluence.

2.4 Transient transfection

The *Anopheles gambiae* Sua 5.1* cell line were transfected with the pGL3-*CYP6M2*_Tias & pGL3-*CYP6M2*_Kis constructs using Qiagen Transfectene reagent according to the manufacturer's recommendations. Briefly, 1.8 µg of the pGL3-*CYP6M2*_Kis and pGL3-*CYP6M2*_Tias were dried down and re-suspended in 6 µL sterile water to allow for wells to be transfected in triplicate. For each well, 600 ng of the plasmids were added in a 2 µL volume. Control constructs were either pGL3-Basic vector without insert (no inherent promoter activity but luciferase gene present) or the shuttle construct (pSLfa1180fa) vector which contains no luciferase gene. Prior to transfection, growth medium was carefully removed, and then 800 µL of sterile PBS (Sigma- Aldrich, St. Luis, MO, USA) was added. The sterile PBS was removed and 350 µL of Schneider's Drosophila medium, 6 µL Effectene (Qiagen, Hilden, Germany) and 4.8 µL Enhancer were sequentially added. Finally, promoter constructs were added (600 ng in 60 µL DNA condensation buffer, containing 1 ng of Actin Renilla plasmid (used to normalize for transfection efficiency). Transfected cells were incubated at 28°C for 24 h prior to the insecticide challenge.

2.5 Cell challenges

In order to check for induction of the *CYP6M2* gene promoter by insecticide, twenty-four (24) hours after transfection, the cells were stimulated with 100 µL Schneider Drosophila medium supplemented with the insecticide permethrin (FMC Corp., Philadelphia, PA, USA) diluted in methanol (ReAgent chem. Cheshire, UK) to a final concentration of 40 µM (High concentration) and 10 µM (Low concentration) per well respectively (Table 1). Schneider's Drosophila medium supplemented with 4% methanol was used as control. These selected concentrations (40 µM, 10 µM and 4% methanol as control) had been used previously [51]. *CYP6M2* gene promoter activity was examined quantitatively by dual luciferase reporter assay (Promega, Madison, WI, USA). Controls had to be treated appropriately to ensure only the desired compound was inducing or repressing promoter activity.

2.5.1 The effect of (40 µM and 10 µM) permethrin and 4% Methanol on promoter activity using pGL3-*CYP6M2*_Tias, pGL3-*CYP6M2*_Kis and pGL3_*CY9M10*_ISOP450 (control)
In order to establish whether activity of the putative *CYP6M2* promoters (pGL3-Kis 896 bp and pGL3-Tias 930 bp) and pGL3-*CYP9M10*_ISOP450 (1504 bp) with a known promoter activity [52] could be induced by insecticide exposure, *Anopheles gambiae* Sua 5.1* cell lines were transfected with the two different luciferase reporter constructs and stimulated with 40 µM and 10 µM concentrations of permethrin and 4% methanol in triplicate.

2.6 Passive Cell lysis
Briefly, Twenty four hours (24 hr) after the cell challenge, growth media was removed and cells were washed with 800 µL of sterile PBS (Sigma Aldrich, USA), then harvested in 100 µL of 1 X Passive Lysis Buffer (PLB) (Promega, Madison, WI, USA) (squirted on to aid dislodging of cells) and placed on a rocking platform for 20 min at room temperature for cell lysis. The cell lysate (8 µL) were transferred into 1.6 mL micro tubes and used for the dual luciferase assay.

2.7 The Dual-luciferase reporter assays
The Dual-Luciferase Reporter (DLR) Assay System from Promega was used to examine promoter activity of the pGL3-*CYP6M2*_Kis and pGL3-*CYP6M2*_Tias constructs. The Renilla plasmid under the control of an actin promoter [52] was co-transfected to achieve Renilla luciferase gene expression driven constitutively to serve as an internal reference control for transfection efficiency (Promega, Madison, WI, USA). Briefly, 24 hours after the cell challenge, 40 µL of Luciferase Reagent II (LARII) was injected into 8 µL lysate and luciferase activity measured for 10 sec on a luminometer (EG & G Berthold, Bad Wildbad, Germany). Then, 40 µl of *Stop and Glo*® reagent whose application quenches firefly luciferase activity thereby allowing bioluminescence produced by Renilla luciferase to be examined and measured (Promega, Madison, WI, USA) was injected to the lysate to stop the luciferase activity and catalyse the Renilla reaction, incubated and then Renilla activity was measured for 10 sec. Luminescence of firefly luciferase was normalized by luminescence of *Renilla* luciferase. The normalized firefly luminescence represented the promoter activity driven by the *CYP6M2* putative promoter Three independent transfections (high permethrin, low permethrin and control, done side-by-side) of three replicates for each promoter construct (n = 9) were conducted.

2.8 Analysis of the 5' upstream of *CYP6M2* gene
In order to predict the putative Sp1 (D-Sp1) binding sites within the cloned and sequenced pGL3-*CYP6M2*_Kis and pGL3-*CYP6M2*_Tias gene promoters, an online analysis using ConSite (http://asp.ii.Uib. no :8090/cgi-bin/CONSITE/consite/) was performed. The *CYP6M2* sequence was also analysed for the presence of CCAT sequence as a potential repressor [560].

3. Results

3.1 Cloning of putative promoter elements upstream of the *CYP6M2* gene for reporter assay measurement
To undertake functional analysis of the regulation of *CYP6M2* expression, we sub cloned the 5' flanking regions of two strains of *Anopheles gambiae* (previously cloned into pJET and called pJET-Tias (930 bp) and pJET–Kis (896 bp) [48] into the luciferase expression vector pGL3-Basic and used these in dual-luciferase reporter assays.

3.2 The effect of (40 µM and 10 µM) permethrin and 4% Methanol on promoter activity using pGL3-*CYP6M2*_Tias, pGL3-*CYP6M2*_Kis and pGL3_*CY9M10*_ISOP450 (control)
Figure 1 shows no increase in relative luciferase activity in cells transfected with pGL3-Basic-*CYP6M2*_Kis and pGL3-*CYP6M2*_Tias compared to the respective pGL3-Basic vector controls (negative control) with/without insecticide challenge. The minimal activity in the promoter less pGL3-Basic indicates the background levels of reporter activity.

Experimental reporter activity levels below this background level are indicative of repression of activity in experimental samples. The pSLfa1180fa shuttle vector is a plasmid with no luciferase gene in and controls for reporter activity and transfection efficiency of the plasmids. The minimal reporter activity seen is likely a reflection of background activity since normalised activity is negligible when compared to activity reported in a previous study [52]. There is limited evidence that pGL3-*CYP6M2*_Kis with no insecticide has higher luciferase activity than controls, however the increase in activity is marginal when compared to other studies [52]. The 5' flanking region of *CYP9M10* has a significantly higher reporter activity level than pGL3-*CYP6M2*_Tias and pGL3-*CYP6M2*_Tias. There is stronger promoter activity for pGL3-*CYP9M10*_ISOP450-HI than pGL3-*CYP9M10*-ISOP450_LI indicating that permethrin may induce activity of this promoter [52].

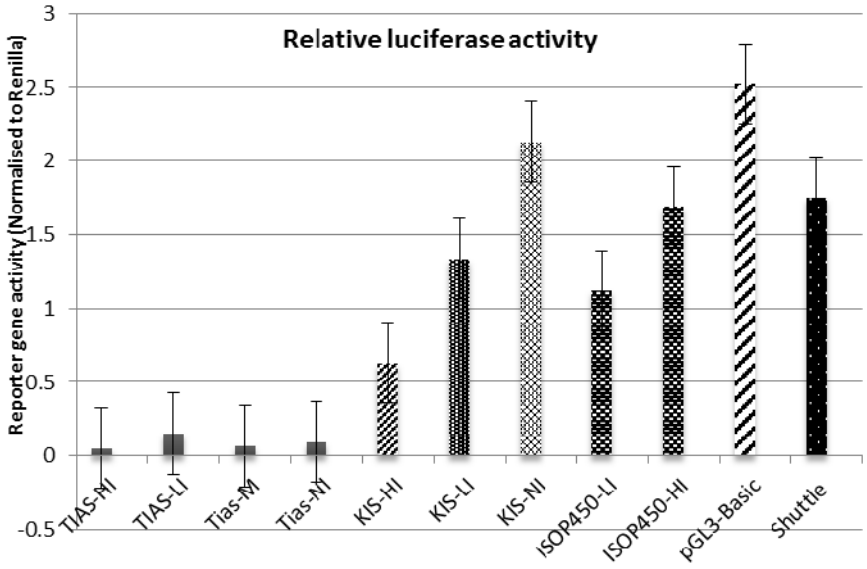

Figure 1: Effects of 4% Methanol, 40µM and 10 µM Permethrin on promoter activity. The normalised luciferase activity of *Anopheles gambiae* pGL3-basic-*CYP6M2* promoter region following the transfection of reporter constructs into *Anopheles gambiae* Suakoko (Sua) 5.1* cell lines. pGL3-*CYP6M2*_Tias-NI 930bp) is the Tiassalé strain with no insecticide and no methanol; pGL3-*CYP6M2*_Tias-HI-is the Tiassalé strain with 40 µM Permethrin, pGL3-*CYP6M2*_Tias-LI- is the Tiassalé strain with 10 µM permethrin. While pGL3-*CYP6M2*_Kis-NI-is the Kisumu strain with no methanol and no permethrin, Kis-HI- Kisumu strain with 40 µM and Kis-LI-Kisumu strain with 10 µM Permethrin. Luc pGL3-basic-ISOP450 (1504 bp) *pGL3CYP9M10* ISOP450-HI is the ISOP450 strain with 40 µM permethrin. pGL3 Basic is the luciferase promoter less vector and pSLfa1180fa (shuttle) is a plasmid with no luciferase gene in and controls for reporter activity in the cells.

3.3 Search for putative Sp1 transcription binding sites within the 896 bp 5' of *CYP6M2*

Table 2: Shows Sp1 putative transcription binding sites within the 5' 896 bp *CYP6M2* at 70% cut off score

Transcription factor	Putative_SP1_binding_sites_within_5'_896_bp_CYP6M2				
	Sequence	From	To	Score	Strand
SP1	CCCACGACCC	4	13	3.900	-
SP1	TAGCCGGGCT	48	57	4.651	+
SP1	ACTGAGCCGC	65	74	4.596	-
SP1	TCACAGCACG	80	89	3.343	-
SP1	TCACTGCGCA	142	151	4.008	-
SP1	ATGGCATTGT	322	331	5.734	+
SP1	CTCACACCCC	345	354	3.807	-
SP1	TCACACCCCC	346	355	7.352	-
SP1	ACACCCCCAA	348	357	7.198	-
SP1	TCTAACCCTC	436	445	3.541	-
SP1	AACCCTCCTC	439	448	4.787	-
SP1	ACCCTCCTCA	440	449	5.550	-
SP1	TCAATACCAC	447	456	4.148	-
SP1	TTCGCACCCC	501	510	5.078	-
SP1	TCGCACCCCG	502	511	3.952	-
SP1	ACCCCGTTCC	506	515	4.600	-
SP1	TTCCCACCGT	512	521	3.893	-
SP1	ATAGCACCCC	577	586	5.078	-
SP1	AGCACCCCCC	579	588	7.718	-
SP1	GCACCCCCCG	580	589	4.750	-
SP1	ACCCCCCGGG	582	591	4.219	-
SP1	ACACCACGCT	595	604	3.708	-
SP1	ACCACGCTGG	597	606	4.219	-
SP1	ACGGTGGGAT	614	623	5.202	+
SP1	CGCGCACGGT	670	679	4.219	+
SP1	ATCCTGACAC	698	707	3.986	-
SP1	TTGGCGTGGC	716	725	5.261	+
SP1	GTGGCGTTGG	721	730	4.750	+
SP1	AAGGTGCGAT	740	749	4.126	+

Table 2 shows a total number of 29 Sp1 (D-Sp1) putative transcription binding sites at different positions within the 896 bp of sequence 5' of *CYP6M2* at 70% cut off score within the *Anopheles gambiae* PEST sequence. The Sp1 (D-Sp1) binding sites are potentially important in the transcriptional activity of the *CYP6M2* gene [56].

3.4 Identification of CCAT Repeat sequence within the 5' upstream of *CYP6M2*

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  1 GAACCCACGA CCCTGAGATT AAGAGTCTCA TGCTCTACCG ACTGAGCTAG 50
 51 CCGGGCTGTT GATAACTGAG CCGCTCGTTT CACAGCACGT TAGCTAACAG 100
101 ACAGCATGAG AGATCTTCAT GCGCACACGT TTTGCGACGC ATCACTGCGC 150
151 AGCCGCAAGA AGGCAAGGGC GTCTAAAGGT AGATTGAAGC CGATGCTTGA 200
201 GATAAGAAAA AATCAGTTAC AGTGTTTATT ATTACCTACA GATTTGAATC 250
251 ATAAACAGTT TCAGTTACTT TATCAATAAA AATATGCATC TATTACTTAC 300
301 TGCATTTATT CGTTATGTTC CATGGCATTG TTCCTATCAA TAAACTCACA 350
351 CCCCCAAAAC GTTAGCTTTC TAACCTTCGC GTATGTTTAC ATATTACTCA 400
401 CCACATTCCT ATGTTTGATC TACGCACACC CTTCTCTAAC CCTCCTCAAT 450
451 ACCACAAGCT CAATTTGATA CACTTCAAGA TTTATGCCTA TGCCATCGTT 500
501 TCGCACCCCG TTCCCACCGT TCACTTGCAC CTTCTTCTTA ATCTTATCTT 550
551 TTTTTATATT TTGTGCAAAC GACTCATAGC ACCCCCCGGG AATACACCAC 600
601 GCTGGACTCT TCACGGTGGG ATCATGCTTA CTTTATTGAG TACACACGCA 650
651 GCGAACTATC AGCGTCGTCG CGCACGGTGA ATGAAACAGT CAACTCATCC 700
701 TGACACACAC GGAATTGGCG TGGCGTTGGC GCAAAAAAAA GGTGCGATAA 750
751 GATTGGGACG AATGTGCAAA AAGAGAAGAC TTGTTGTTTT GGCTTCTAAT 800
801 TCGCCCTATA AAGAGAACCG GTTTGAGTGA CCTCCATCATCAGTTGTCGG  850
851 TGGACAGTCA AATCAATCGA ACGTGGTGCT CCTCGCGTTC CAAAAA     896
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Figure 2: Sequence of cloned 896 bp fragment of 5' flanking region of *CYP6M2* gene showing putative TATA box and CCAT motifs. There is a TATA box at –203 bp and two putative CCAT boxes at – 265 bp and –439 bp relative to the transcription start site and these CCAT in a promoter sequence are considered as repressor sequences.

Figure 2 shows the presence of 3 copies of CCAT sequence within the 5' upstream *CYP6M2* gene. CCAT motifs are potential repressors of transcription [56]. The CCAT sequences are in positions -265 bp, -310 bp and -439 bp relative to the transcription start site.

4. Discussion

The emergence of insecticide resistance due to widespread use of insecticides has become a major public health problem, which presents a great challenge to the control of vector borne diseases [2, 53]. Here we studied *CYP6M2*, a member of the Cytochrome P450 super family, because of its potential role in the metabolism of insecticides during xenobiotic exposure. Our interest lies in understanding the regulatory mechanism involved in the control of *CYP6M2* expression, particularly as its over expression can result in insecticide resistance [18, 26] . In the current study, we detected no promoter activity using the Dual luciferase assay, with some indication of a reduction in luciferase activity in the presence of the *CYP6M2* promoter possibly indicative of the presence of repressor sequence (Figure 1 and 2). Whilst we have no experimental evidence for the presence of repressor motifs within the sequence upstream of *CYP6M2*, we note that Sp1 (D-Sp1) binding sites may initiate repressor activity [54] and in this study, a total of 29 putative Sp1 (D-Sp1) were identified (Table 2) throughout the 896 bp of sequence upstream of *CYP6M2*. Repression is mediated by proteins that can operate in a dedicated or facultative manner to block transcription via mechanisms that include direct interaction with the basal transcriptional machinery or with chromatin-modifying machinery (Figure 3) [55]. Sp1 (D-Sp1) has been shown to recognize and specifically bind to GC-rich sites within the simian virus 40 (SV40) promoter via three Cys2His2 zinc finger motifs [54] and further study of this is warranted in our system.

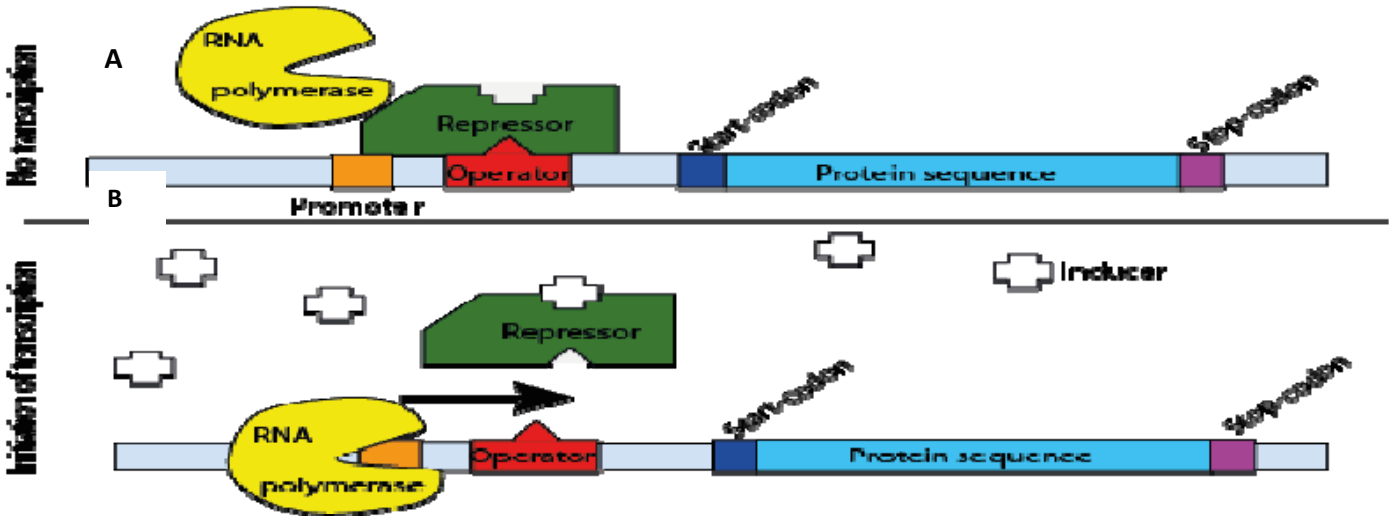

Figure 3. Schematic representation of the repressor sequence. A: when a repressor protein is bound to the operator DNA sequence (which normally lies 5' upstream of the start -codon of the 'protein of interest'. The RNA polymerase therefore is not able to start transcribing the protein sequence. **B:** When an inducer is added the repressor protein detaches from the operator and subsequently the RNA polymerase congregates with the promoter and the protein now becomes transcribed into mRNA (Adopted and modified from Balzer *et al*., 2013).

Figure 2 shows the presence of CCAT repeat sequences within the 5' upstream region of *CYP6M2* and the presence of these sequences may be another potential reason for the lack of promoter activity. Earlier studies have shown that four repeats of CCAT sequence are sufficient to repress the *CD30* promoter in humans [56]. Again, further study of this is warranted, perhaps through side directed mutagenesis of CCAT motifs prior to dual luciferase assays. Our data indicate that the sequence upstream of *CYP6M2* does not have measurable promoter activity and, in fact, appears to repress activity. This is counter to expectations given the upregulation of *CYP6M2* frequently seen in studies of insecticide resistant *An. gambiae* including of the Tiassalé strain [26]. Further experimentation is required to resolve this but we hypothesize either that promoter elements are present but over-ridden by the presence of strong repressors (deletion constructs of the cloned 5' upstream region may remove repressor elements and so identify the promoter elements) or that, given *CYP6M2* is part of a cluster of P450 genes, that they are under the control of a single promoter upstream of the cluster and that the region studied, directly upstream of *CYP6M2*, does not have gene-specific promoter activity.

5. Conclusion

This work provides a first step in identifying the regulatory mechanisms involved in the expression of *CYP6M2*. We identify two potential regulatory elements, Sp1 (D-Sp1) and CCAT repeats sequences which may be responsible for repression of expression although further work will be required to elucidate the exact roles played by both Sp1 (D-Sp1) and CCAT regions in the repression of induction of *CYP6M2*. Our work also indicates that the promoter of *CYP6M2* may not be directly upstream of the gene but may lie 5' of the cluster of *CYP6* genes of which *CYP6M2* is a part. Additional experimental work will be required to investigate this.

6. Acknowledgments

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