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Identification of the transcription initiation site reveals a novel transcript structure for *Plasmodium falciparum maebl*

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Abstract

Strict regulation of gene expression is critical for the development of the malaria parasite within multiple host cell types. However, much remains unexplored regarding gene regulation in *Plasmodium falciparum* with only a few components of the gene regulation machinery identified thus far. Better characterization of transcript structures with precise mapping of transcript ends will greatly aid in the search of conserved regulatory sequences in the genome. Transcript analysis of *maebl*, a member of the *ebl* gene family, in *P. falciparum* intra-erythrocytic stages has revealed a unique transcript structure for *maebl*. The 5′ untranslated region of *maebl* transcript is exceptionally long (>2 kb) with a small multi-exon open reading frame, annotated as a putative mitochondrial ATP synthase (PF11_0485) in the *Plasmodium* database. Northern blot hybridizations and RT-PCR analysis confirmed a bicistronic message for *maebl* along with PF11_0485. We further identified the minimal *maebl* promoter to be upstream of PF11_0485 by using transient chloramphenicol acetyl transferase (CAT) reporter assays. The occurrence of a bicistronic mRNA in *Plasmodium* is both novel and unusual for a lower eukaryote and adds on to the complexity of gene regulation in malaria parasites.

Index descriptors

Plasmodium falciparum; malaria; *maebl*; gene regulation; promoter; bicistronic mRNA

1. Introduction

Malaria parasites must invade and multiply within different cell types to sustain their life cycle. Through the course of development in both the vertebrate and the invertebrate hosts, malaria parasites exhibit a strictly regulated pattern of gene expression. Whole-genome transcription profiles have been extremely valuable in providing insights into levels and timing of gene expression in *Plasmodium* (Bozdech, et al., 2003, Le Roch, et al., 2003). The

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unavailability of transcript structure for most genes however complicates the precise identification of regulatory regions in the *Plasmodium* genome.

Identifying conserved regulatory motifs is further hindered in *Plasmodium* by its AT-rich genome and the presence of extensive repeat sequences. Some recent bioinformatic studies have hence used special algorithms that take into account the unique genetic composition of *Plasmodium* and identified conserved regulatory motifs in different categories of genes (Young, et al., 2008) (Wu, et al., 2008). Most *Plasmodium* promoters also contain an over-representation of homopolymeric tracts (dA:dT) (Horrocks, et al., 1998), which could contribute to transcription factor binding and transcriptional regulation as in other organisms (Hori and Firtel, 1994, Struhl, 1985, Winter and Varshavsky, 1989). Although *Plasmodium* lacks many components of the conserved eukaryotic transcription machinery, the recent characterization of ApiAp2-mediated regulation of gene expression identifies a conserved mechanism of gene regulation in apicomplexan parasites and plants (De Silva, et al., 2008).

maebl is a paralogue of the *ebl* gene family in *P. falciparum* that encodes erythrocyte-binding ligands EBA175 and BAEBL (EBA140). *maebl* transcripts are found in mid-trophozoite stages during intra-erythrocytic development and are expressed at maximum levels in the mosquito midgut sporozoite stages (Blair, et al., 2002, Le Roch, et al., 2003). *maebl* stands out as a classic example of complex gene structure in *Plasmodium*, with alternative splicing creating distinct MAEBL isoforms (Saenz, et al., 2008, Singh, et al., 2004). In this study, we further analyzed *maebl* in *P. falciparum* erythrocytic stages by characterizing its transcript structure and identifying its promoter sequences.

2. Materials and Methods

2.1. Parasite culture and maintenance

Clones of *Plasmodium falciparum* 3D7 were obtained from the Naval Medical Research Center and maintained in culture according to standard methods at 37 °C and gassing (5% O_2, 5% CO_2, Nitrogen balanced) with 5% hematocrit in RPMI 1640 (Invitrogen) supplemented with 0.5% Albumax I (Invitrogen), 0.25% sodium bicarbonate and 0.01mg/ml gentamicin. Human red blood cells were obtained from Indiana Blood bank and washed three times with RPMI 1640 (Invitrogen), resuspended to 50% hematocrit and stored at 4°C.

2.2. Genomic DNA and RNA extraction

Plasmodium falciparum genomic DNA was purified from blood-stage parasites using a standard phenol/chloroform method as described before (Balu, et al., 2005). Total RNA was isolated using TRI REAGENT (Molecular Research Center, Inc) RNA isolation protocol and mRNA was then isolated by purification through an oligo(dT) cellulose column.

2.3. Northern blot hybridizations and RT-PCR

Two μg of *P. falciparum* 3D7 total RNA was separated by formaldehyde gel electrophoresis along with a 0.5 kb-10 kb RNA ladder (Invitrogen). The gel was then blotted by standard methods on to a nylon membrane and hybridized overnight at 65°C with respective ^{32}P-labeled probes for *maebl* (PCR amplified from genomic DNA with JA-503 and JA-504) and

PF11_0485 (PCR amplified from genomic DNA with JA-739 and JA-741) in 10 ml of 0.1 M disodium hydrogen phosphate with 1% SDS. The blot was washed three times at 65°C with washing solution (0.3 M disodium hydrogen phosphate; 1 mM EDTA; 1% SDS), dried and exposed to an X-Ray film for 2 days at −80°C. RT-PCR for the entire 5′ region of *maebl* was performed using the one-step RT-PCR kit (Invitrogen) with primers JA-740 and JA-712 (Table 1).

2.4. Random amplification of cDNA ends (RACE)

The SMART™ RACE cDNA amplification kit (Clontech) was used to identify the transcript ends of *maebl* by performing 5′ RACE and 3′ RACE. The gene-specific primers used for 5′ RACE of *maebl* were JA-650 and JA-651 (Table 1). The primer used for 3′ RACE of *maebl* was JA-676 (Table 1). The conditions for the PCR were 94°C for 1 min followed by 35 cycles of 15 s at 94°C; 30 s at 49°C; 1 min at 65°C.

2.5. PCR amplification, cloning and sequencing

The primer sequences used in all the cloning experiments are summarized in Table 1. Two different regions in the 5′ sequences of *maebl* were amplified from *P. falciparum* 3D7 genomic DNA as follows: mp: JA-837 and JA-838; 35 cycles of 15 s at 94°C; 30 s at 45°C; 1 min at 65°C; wm: JA-837 and JA-1433; 35 cycles of 15 s at 94°C; 30 s at 45°C; 2 min at 65°C. The coding sequence of *chloramphenicol acetyl transferase* (*cat*) was amplified from the vector pCAT (Promega) by using primers JA-1220 and JA-1221. The PCR conditions were 35 cycles of 15 s at 94°C; 30 s at 48°C; 1 min at 65°C. The PCR products and the RACE PCR-amplified products were cloned into the pCR-2.1 vector by using the TOPO-TA cloning kit (Invitrogen) and sequenced using M13 forward and reverse primers.

2.6. Plasmid constructs

p11zCH: 3′ *hsp 86* was amplified as a KpnI/Hind III from *P. falciparum* 3D7 genome using primers JA- 635 and JA- 636 and T-cloned into pCR2.1 (Invitrogen). Chloramphenicol acetyl transferase coding sequence was amplified as a BamHI/KpnI fragment from the plasmid vector pCAT (Promega) and cloned into pCR2.1 upstream to 3′ *hsp 86*. The *cat* coding sequence along with 3′ *hsp 86* was excised as a BamHI/HindIII fragment from pCR2.1 and cloned into the vector pGEM-11zf(+) (Promega). p11zCH-mp and p11z-CH-wm: These reporter constructs for *maebl* were created by cloning the two different regions of *maebl* 5′ sequences into the vector p11zCH using XhoI and BamHI.

2.7. Exonuclease digestions

Unidirectional exonuclease digestions were performed on the *maebl* reporter plasmids by using the Erase a Base System (Promega). In brief, plasmid DNA was digested first with AatII and then with Eco01019 and exonuclease digestions were performed according to the manufacturer's protocol at 22°C and the plasmids containing deletions were then sequenced to identify the deleted sequences.

2.8. Transient transfection of *Plasmodium falciparum* and Chloramphenicol acetyl transferase (CAT) reporter assays

Plasmodium falciparum 3D7 parasites were transfected as described previously (Wu, et al., 1995). Briefly, *Plasmodium falciparum* ring stage parasites were synchronized with 5% sorbitol in RPMI 1640 and after one generation of growth, the parasite culture was split into four equal volumes for transfection such that equal number of parasites were transfected with the four different plasmids pHC1CAT, p11zch-mp, p11zch-Δmp1 and p11zch-Δmp2. Parasites were electroporated at 0.31 KV and 950 μF capacitance in 0.2 cm cuvette using a Bio-Rad gene pulser III and reintroduced into a 5 ml culture at 5 % hematocrit. CAT assays were performed 48 hrs later at young trophozoite-stages as described previously (Balu and Adams, 2003) using a FAST CAT green substrate reagent (Invitrogen).

3. Results

3.1. Transcript analyses of *maebl*

Northern hybridization with a probe in the 3′ coding region of *maebl* identified a transcript slightly greater than 8 kb for *maebl* (Figure 1A). The coding sequence for *maebl* is approximately 6.2 kb in length, indicating that *maebl* untranslated regions (UTRs) were around 2 kb long. In order to characterize the individual lengths of 5′ and 3′ UTRs of *maebl*, random amplification of cDNA ends (RACE) was performed on *maebl* transcripts. Multiple transcription start sites, close to each other, were identified for *maebl* at 2163 bp, 2139 bp, 2073 and 1937 bp upstream of the translational start codon. (Figure 1B). No RACE products were obtained while using different sets of primers near the start of *maebl* coding sequence (data not shown) probably due to the inefficiency of the method to amplify greater than 2kb sequences. Two transcription stop sites were identified for *maebl* at 191 bp and 267 bp downstream of the translational stop codon by using 3′ RACE (Figure 1B).

3.2. A possible bicistronic transcript for *maebl*

Sequence analysis of *maebl* 5′ UTR revealed a multi-exon open reading frame, designated as a mitochondrial ATP-synthase subunit (PF11_0485) in the *Plasmodium* database (PlasmoDB, ver. 5.4) (Kissinger, et al., 2002) that is well conserved in different *Plasmodium* species (Figure 1B). To investigate whether this open reading frame is a part of *maebl* transcript, we repeated the northern hybridization for *maebl* by using PF11_0485 as a probe and a similar >8 kb transcript was seen suggesting PF11_0485 to be a part of *maebl* transcript (Figure 1C). As further confirmation, RT-PCR was performed with a sense primer (JA-739) at the start of PF11_0485 coding sequence and an antisense primer in the *maebl* coding sequence (JA-712). A 2.1 kb fragment was amplified that was confirmed by sequencing to contain the spliced PF11_0485 transcript, the intervening 1.3 kb region and the beginning of *maebl* transcript (Figure 1D).

3.3. Identification and characterization of *maebl* promoter

To identify *maebl* promoter, two different 5′ regions of *maebl* were cloned into a chloramphenicol acetyl transferase (CAT) reporter construct, p11z-CH that was created for transient expression in *P. falciparum. maebl* putative promoter (mp)- contained a 329 bp region in 5′ *maebl* from 2303 bp to 1975 bp upstream of *maebl* start codon. Whole 5′ *maebl*

(wm)- contained a 2.3 kb 5′ region of *maebl* from 2303 bp to 1 bp upstream of *maebl* start codon (Figure 2A). Forty eight hours post transfection with the *maebl* reporter constructs, parasite CAT activity was assayed and compared to the positive control plasmid pHC1-CAT (Crabb and Cowman, 1996). Parasites transfected with the 5′ region 'mp' showed significant CAT expression confirming its promoter activity whereas those transfected with the whole 5′ region of *maebl* showed very little promoter activity (Figure 2B). To identify the minimal promoter region within 'mp', unidirectional exonuclease digestions were performed on the plasmid p11z-CH-mp resulting in two 5′ deletions. The first deletion removed −2303 bp to −2112 bp from mp and the second deletion removed −2303 to −2053 bp giving raise to p11z-CH-Δp 1 and p11z-CH-Δmp 2, respectively. Both these deletions significantly reduced the promoter activity of 'mp', suggesting that the 5′ region from -2302 bp to -2112 bp contains maximum promoter activity (Figure 2B). A closer look at the sequences in this promoter region showed a poly (dA) repeat and an $(ATT)_{10}$ tri nucleotide repeat (Figure 2C) that have been previously described in promoter regions of other organisms.

4. Discussion

A member of the *P. falciparum ebl* gene family of ligands, *maebl*, serves as a classic example of complex gene regulation mechanisms in this malaria parasite. A cDNA clone from the rodent parasite *P. yoelii* previously identified the 5′ UTR to be approximately 1.8 kb long (Kappe, et al., 1998). Although uncommon, such long 5′ UTRs have been reported in *Plasmodium* (Militello, et al., 2004, Myrick, et al., 2003, Porter, 2001). *maebl* is also alternatively spliced in different *Plasmodium* species that results in different possible isoforms of the protein (Singh, et al., 2004). In this study we intended to further characterize the transcript structure of *maebl* in *P. falciparum* blood stages and identify its promoter sequences.

By using RACE, multiple transcription start sites were mapped for *maebl* with the farthest start site located approximately 2.1 kb upstream of the translational start codon. Even though RACE is known to yield some false-positive results, the close location of the identified transcription sites strongly suggests the actual start site for *maebl* to be in this region. Two transcription stop sites were mapped for *maebl* using 3′ RACE at around 0.2 kb and a consensus eukaryotic polyadenylation signal ATTAAA was found 15 bp before the second stop site.

Sequence analysis of 5′ UTR of *maebl* identified a 0.7 kb region in the 5′ UTR that is conserved in *P. falciparum* and the rodent parasites, *P. yoelii* and *P. berghei* and contains a small predicted open reading frame (PF11_0485) of 465 bp that spans two introns. Northern blot hybridizations using a probe corresponding to PF11_0485 and RT-PCR analysis confirmed a bicistronic message for PF11_0485 and *maebl*, which has not been reported in *Plasmodium* thus far. In bicistronic mRNAs of other organims, ribosomes re-initiate translation at the downstream start codon and the re-initiation efficiency increases with an increase in the length of the inter-cistronic region and a decrease in the length of the upstream ORF (Kozak, 1987). Both these scenario correspond well to PF11_0485 and *maebl*, where the inter-cistronic region is approximately 1.3 kb and the upstream gene, PF11_0485, codes for a short open reading frame. The presence of this bicistronic message

for *maebl* in other parasite life cycle stages as well as the functional importance of its product remain to be investigated.

Two different 5′ regions of *maebl*: a small 329 bp region just upstream to PF11_0485 (mp) and this region along with the rest of the 5′ UTR (wm), were tested for promoter activity by transient CAT assays in *P. falciparum*. The 5′ region 'mp' displayed significant promoter activity in the parasite blood stages and loss of CAT expression following exonuclease digestions on this region, further deduced the promoter activity to the first 192 bp of this region. The striking features in this promoter region were the presence of long homopolymeric (dA:dT) tracts that have been shown to be present in *Plasmodium* promoters previously (Horrocks, et al., 1998) and $(ATT)_{10}$ tri nucleotide repeats that have been previously reported in other organisms to cause DNA instability and are known to play a role in gene regulation by allowing interactions with gene regulatory proteins (Stallings, 1994, Trotta, et al., 2000). However, an exhaustive analysis of the 5′ regions in the genome will be required to confirm the prevalence of these structures in *Plasmodium* promoters. Significant reduction in promoter activity with the entire 5′ region of *maebl* (wm) suggests the presence of possible silencing elements. However, several regions of 5′ *maebl* would need to be analyzed individually to identify such regions with possible enhancer/silencer activity.

In summary, *maebl* is transcribed along with the upstream gene, PF11_0485, as a bicistronic transcript and a minimal 5′ region with promoter activity was mapped upstream to PF11_0485 and the mapped transcription start sites. Our study on the transcript structure of *P. falciparum maebl* has thus revealed another novel and intriguing aspect of *Plasmodium* gene regulation that further adds to the uniqueness of this parasitic protozoan.

Acknowledgments

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Abbreviations

CAT	chloramphenicol acetyl transferase
EBA175	erythrocyte binding antigen-175
EBA140	erythrocyte binding antigen-175
RACE	random amplification of cDNA ends

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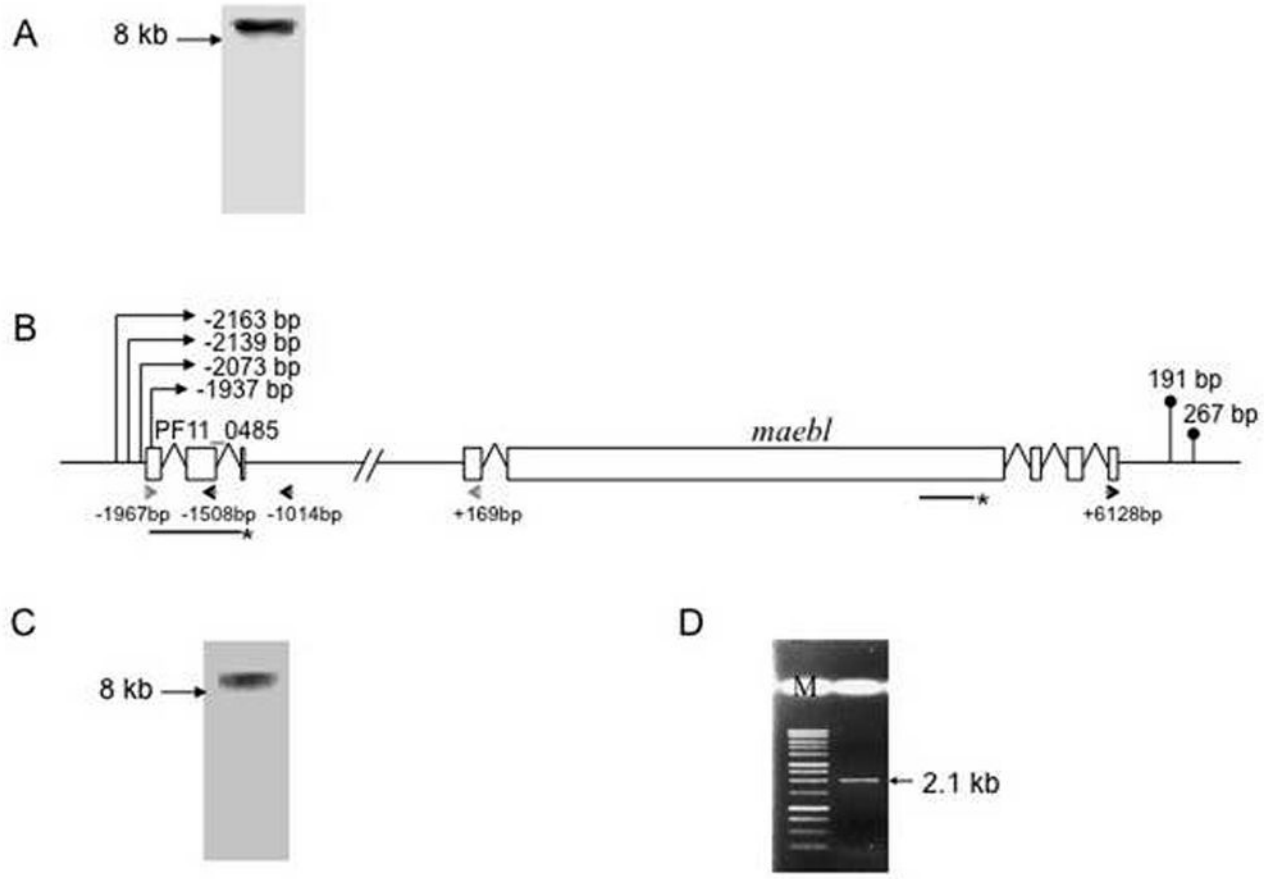

Fig. 1. Transcript analysis of *P. falciparum maebl*

A. Northern hybridization with a probe in the 3′ region of *maebl* identified a slightly >8kb transcript for *maebl*. B. A schematic representation of *maebl* gene structure with the identified transcription start and stop sites and the location of primers and probes (marked with *). Four transcription start sites and two stop sites were mapped for *maebl* using RACE. Gene specific primers used in RACE are shown as single black arrows. A conserved, multi-exon open reading frame (ORF), annotated as PF11_0485, is present in the 5′ untranslated region of *maebl*. C. Northern hybridization performed on *P. falciparum* 3D7 total RNA with PF11_0485 coding sequence as a probe identified a >8 kb transcript similar to that seen with the *maebl* probe, thereby suggesting a bicistronic transcript for *maebl* and PF11_0485. D. RT-PCR was performed on *P. falciparum* 3D7 total RNA with a sense primer in the first exon of PF11_0485 and an antisense primer in the *maebl* coding sequence (shown as single grey arrows). The 2.1 kb product was confirmed by sequencing to contain *maebl* along with the intervening 1.3 kb region and the appropriately spliced PF11_0485 coding sequence (M-marker).

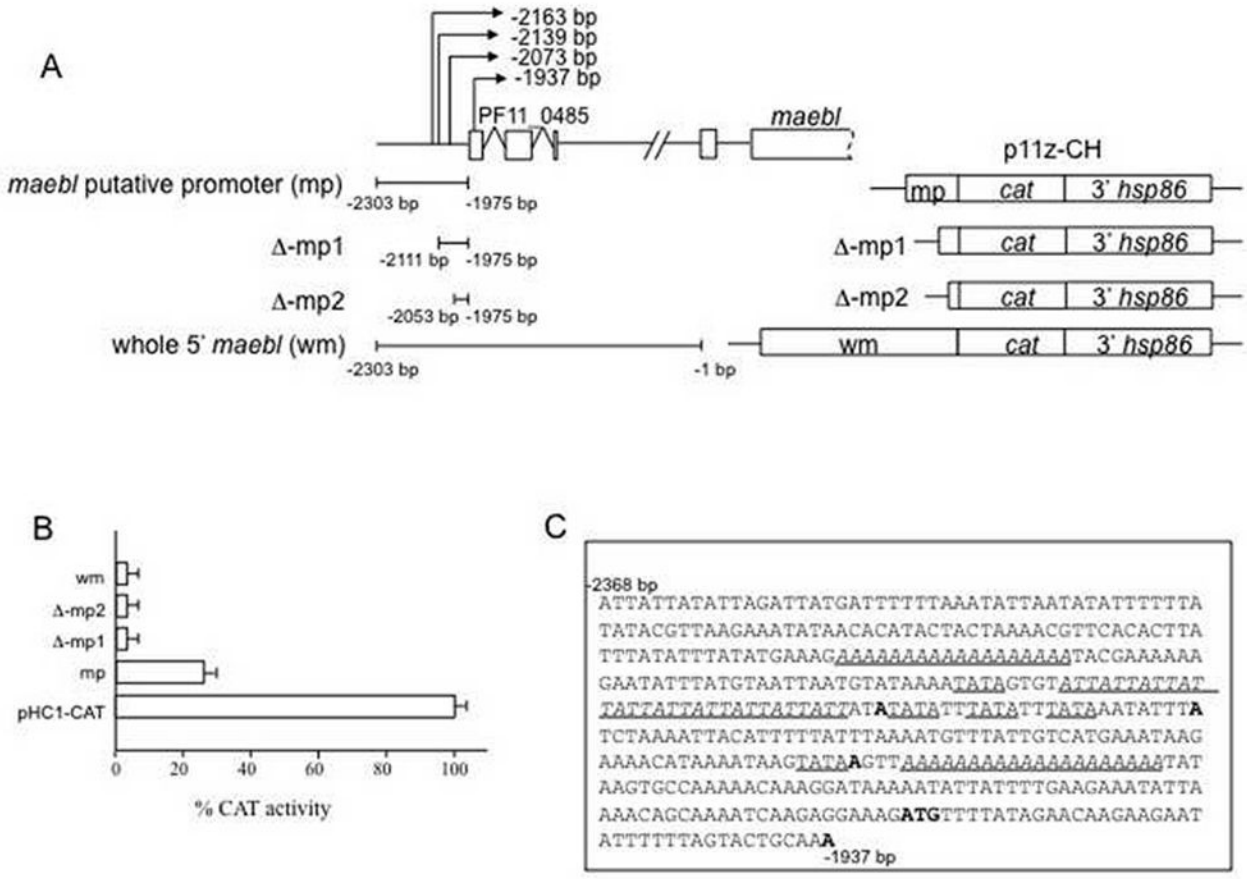

Fig. 2. Reporter assay analysis identifies possible promoter region of *P. falciparum maebl*

A. Two different regions from 5′ *maebl* were first tested for promoter activity. *maebl* putative promoter (mp) consisted of a 329 bp region upstream of PF11_0485. Whole 5′ *maebl* (wm) contained mp along with the rest of the 5′ UTR of *maebl*. The two *maebl* 5′ regions were cloned individually into a chloramphenicol acetyl transferase (CAT) reporter plasmid, p11z-CH, created for transient assays in *P. falciparum*. Unidirectional exonuclease deletions on mp resulted in Δ-mp1 and Δ-mp2. B. Forty eight hours post-transfection, the parasite cell lysate was assayed for CAT expression. CAT activity was compared to the control plasmid pHC1-CAT that contained a strong, constitutive *calmodulin* promoter. The 5′ region ‘mp’ around the transcription start sites showed significant promoter activity that was greatly reduced in the deletions Δ-mp1, Δ-mp2. The 5′ region ‘wm’ also displayed only slightly detectable promoter activity. C. Sequences around the transcription start sites show the possible *cis* regulatory motifs in 5′ *maebl* (italicized and underlined). Homopolymeric (dA) tracts are seen along with $(ATT)_{10}$ trinucleotide repeats, which are known to cause structural changes in the DNA. Some TATA boxes are also seen around the start sites. Shown in bold are the four transcription start sites mapped for *maebl* and the start codon of PF11_0485.

Table 1

List of primers used in RACE and cloning experiments.

Primer	Sequence
JA-503	5′-AATAACATGAAAGGAAATAATAATG-3′
JA-504	5′-TGAAAATTCCTTTTTTTTAAAAC-3′
JA-635	5′-ATAGGTACCGGATTTATATAATATATTTATG-3′
JA-636	5′-ATAAAGCTTATTAAGGAAACAAAATGAAAG-3′
JA-650	5′-AAGTGAGTTAAAATGTAAAAGAT-3′
JA-651	5′-CTCCATTTATCATCCGTAGCATC-3′
JA-676	5′-GCAATCCTTTAAGTTATTCGGAAG-3′
JA-712	5′-CATATTTGAATTTCCCCTTTCCATGTATCCCAGAGTTTG-3′
JA-739	5′-GAACAAGAAGAATATTTTTTAGTACTGC-3′
JA-741	5′-GGCATGCATGGAGAACTACC-3′
JA-817	5′-CCAACATGTGTACTGAAAAAGG-3′
JA-818	5′-GGGGTCATACTCCTTCATGG-3′
JA-837	5′-ATACTCGAGCACATACTACTAAAAACGTTCACAC-3′
JA-838	5′-ATAGGATCCCATCTTTCCTCTTGATTTTGCTG-3′
JA-1220	5′-ATAGGATCCCAGGTTCAATACAGCTCTTAAG-3′
JA-1221	5′-ATAGGTACCCTTATAATGTCTGCTCGAAGC-3′
JA-1433	5′-CGGGATCCATTTATTACAATAAAATAAAAGAATATG-3′