A conserved U-rich RNA region implicated in regulation of translation in *Plasmodium* female gametocytes

Joanna A. M. Braks, Gunnar R. Mair, Blandine Franke-Fayard, Chris J. Janse and Andrew P. Waters*

Department of Parasitology, Centre of Infectious Diseases, Leiden University Medical Centre (LUMC), Leiden, The Netherlands

ABSTRACT

Translational repression (TR) plays an important role in post-transcriptional regulation of gene expression and embryonic development in metazoans. TR also regulates the expression of a subset of the cytoplasmic mRNA population during development of fertilized female gametes of the unicellular malaria parasite, *Plasmodium* spp. which results in the formation of a polar and motile form, the ookinete. We report the conserved and sex-specific regulatory role of either the 3′- or 5′-UTR of a subset of translationally repressed mRNA species as shown by almost complete inhibition of expression of a GFP reporter protein in the female gametocyte. A U-rich, TR-associated element, identified previously in the 3′-UTR of TR-associated transcripts, played an essential role in mediating TR and a similar region could be found in the 5′-UTR shown in this study to be active in TR. The silencing effect of this 5′-UTR was shown to be independent of its position relative to its ORF, as transposition to a location 3′ of the ORF did not affect TR. These results demonstrate for the first time in a unicellular organism that the 5′ or the 3′-UTR of TR-associated transcripts play an important and conserved role in mediating TR in female gametocytes.

INTRODUCTION

Translational repression (TR) of stored mRNA plays an important and perhaps universal role in the development of the zygote and early embryo and guards the establishment of embryonic polarity in multicellular organisms (1–3). Early embryonic development and polarity are facilitated by the spatio-temporal regulation of distribution and expression of mRNA transcripts, phenomena mediated by multi-protein complexes established in the female germ-line cell that are themselves sensitive to protein kinase activity triggered by fertilization (4–7). Embryos may use TR as a strategy to control gene expression as transcription in the zygote is arrested during the first few hours after fertilization whilst simultaneously requiring developmentally programed responses to changes in the intra- and extracellular environment (8,9). Thus the initial requirements of the zygote for *de novo* protein synthesis are met from a hitherto stored and silent mRNA repertoire resident in the female germ line cell. The mechanisms underpinning TR have been studied extensively in metazoans and generally involve interactions of RNA-binding proteins with specific sequences in the 5′- and more generally 3′- untranslated regions (UTRs) of translationally repressed transcripts (10–12) that influence mRNA stability, accessibility to the translational apparatus and translational capacity. For example, the cytoplasmic polyadenylation element (CPE) and AU-rich elements (ARE) may play roles in mediating transcript stability by influencing the length of the poly(A) tail through stimulating poly(A) addition (CPE) or deadenylation (CPE and ARE) in a context dependent manner (13,14). In addition, various 3′-UTR elements have been identified that bind to proteins that directly or indirectly interfere with the cap-dependent translation initiation process (15).

The significance and mechanisms of TR in the control of gene expression during development of unicellular eukaryotes are relatively less well-studied. *Saccharomyces cerevisiae* are known to reversibly redistribute actively

translated mRNA to P-bodies under conditions of stress (16) and the existence of similar structures has been shown be present in Trypanosomes (17,18). Indeed Trypanosomatids are thought to control gene expression almost exclusively in a post-transcriptional manner and the 3′-UTR in several examples has been shown to be critical in regulating mRNA stability and thus protein production (19). However, a direct parallel of production and storage of specific mRNA species for use during a later program of development is lacking in single cell eukaryotes. Single cell organisms belonging to the genus *Plasmodium* produce cell cycle-arrested, sexual precursor cells (gametocytes) in the blood of the vertebrate host, which are obligatory for transmission of the parasite from the vertebrate host to the mosquito vector. Gametocytes are activated by the environment of the mosquito midgut stimulating gamete formation and fertilization. The zygote develops within 18–24 h into the polarized, motile ookinete that will penetrate the wall of the midgut and truly infect the mosquito vector. Two closely related EGF-domain containing proteins P28 and P25 of *Plasmodium* are abundantly expressed on the surface of the ookinete and play an essential role in development and infectivity (20). Although both proteins are only expressed in the mosquito stage of the parasite, their cognate mRNAs are abundant in female gametocytes, implying that these transcripts are subject to TR (21–23). A comparative analysis of transcriptome and proteome data of the rodent parasite *Plasmodium berghei* life cycle revealed that at least 11 transcripts, including *pb25* and *pb28*, are likely to be subject to TR in gametocytes (24). Furthermore, *pb28* and *pb25* mRNAs in female gametocytes are physically associated with DOZI, a protein with homology to the DDX6 family of RNA helicases that is associated to TR in multicellular eukaryotes. DOZI influences mRNA stability and is involved in the process of silencing of many gametocyte mRNA species (22). The data suggest that TR is a central mechanism for regulation of gene expression during sexual development of *Plasmodium* and shares features with TR in metazoans. A 47 nucleotide (nt) U-rich potential *cis*-acting motif was identified in the 3′-UTRs of a subset of translationally repressed transcripts in *P. berghei* gametocytes using the motif finder program MEME and implicated to be involved in TR (24).

In order to analyze the role of the 3′-UTR and the identified conserved U-rich 47 nt element in TR, we developed a stable GFP reporter system in the rodent parasite *P. berghei*. The effect of a set of different 3′ and 5′-UTRs on TR was tested by determining steady state GFP mRNA and protein levels in the gametocyte stage. We demonstrate that the 3′-UTR is essential for TR of two transcripts, *pb28* and *pb000245.02.0* whereas the 5′-UTR regulates TR of *pb25* transcripts. We show that *trans*-acting factors for TR of *pb28* transcripts are exclusively present in the female gametocyte and that a U-rich RNA-element plays a conserved role in TR. The results demonstrate for the first time the important role of UTRs and confirm the significance of TR as a mechanism of post-transcriptional regulation of gene expression in *Plasmodium*.

MATERIAL AND METHODS

GFP reporter constructs

The GFP expression plasmid PbGFP$_{CON}$ (25) was used to construct a series of *P. berghei* reporter plasmids that direct expression of *gfp* under control of different *Plasmodium* promoter/5′-UTRs and 3′-UTRs. The *ef-1αα* promoter was replaced by different 5′ regulatory regions using the unique EcoRV and BamHI restriction sites. Alternative 3′ regions were cloned in the vector using the XbaI and Asp718 sites. The different 5′- and 3′-UTR fragments were PCR amplified from genomic DNA of *P. berghei* reference clone15cy1 and *P. gallinaceum* strain 8A (a kind gift from Joe Vinetz) using the primer sets as in supplementary Table S1. For construction of the truncated/hybrid *pb28* 3′-UTRs, different 3′-UTR fragments with EcoRV and HindIII sites were generated and combined. The 5′ promoter regions of *pb000652.01.00* (*ccp2*), *pb000609.00.0* (*α-tubulin II*) and *pb000791.03.0* have been described (26).

Transfection and genotype analysis of transgenic parasites

Transfection of *P. berghei* parasites (reference wild type parasites of cl15cy1, ANKA strain), selection and cloning of the transgenic parasites were performed as described previously (27). After linearization at the unique KspI site the reporter constructs described above were transfected separately into *P. berghei* generating transgenic lines as listed in supplementary Tables S2–S4. Correct integration of the constructs into the *ssu-rrna* gene unit was confirmed by Southern analysis of chromosomes separated by Field Inversion Gel Electrophoresis (FIGE) as described (28) and by diagnostic PCRs (25).

3′ RACE experiments

In order to determine the polyadenylation sites of mRNA, total cDNA was synthesized from 5 μg of DNase I-treated total RNA obtained from purified *P. berghei* gametocytes using Superscript III Reverse Transcriptase (Invitrogen) and an oligo(dT) primer (L145B). Reverse PCR was performed with the same oligo(dT) combined with an internal sense primer (for primer list see supplementary Table S1) using the following PCR cycles: 94°C for 2 min, 56°C for 45 s and 68°C for 1 min (40–50 cycles). Products were cloned into pCR2.1-TOPO (Invitrogen) and sequenced.

Collection of gametocytes and ookinetes

To obtain enriched suspensions of mature gametocytes, mice were treated with phenylhydrazine before infection as described (29). Infected blood (1 ml) containing gametocytes was collected by cardiac puncture at room temperature and mixed directly in enriched PBS (20 mM HEPES, 20 mM glucose, 4 mM NaHCO3, 0.1% BSA, pH 7.25–7.30) to prevent activation of gamete formation. Leukocytes were removed as described (30) and subsequently gametocytes were purified using Nycodenz density gradient centrifugation as described (31). Purified gametocytes (50–90% gametocytes mixed with asexual blood stages) were collected in 1–3 ml of enriched PBS and used

for further analysis (see below). Ookinetes were grown in standard small scale *in vitro* cultures using tail blood from the phenylhydrazine treated mice (29).

Western blot analysis

Protein production was detected by western blot analysis according to standard procedures using 12% SDS polyacrylamide gels. Immunoreactions were performed with the following antibodies: rabbit polyclonal anti-Pb28 antisera (no 881 and 882; 1:1,000), mouse monoclonal *P. yoelii* anti-P25 (1:200; a kind gift from Takafumi Tsuboi), mouse monoclonal anti-GFP (Roche, 1:2,000) and rabbit polyclonal antiserum raised against the female gametocyte specific protein P47 of *P. berghei* (22). Antibody reactions were visualized by ECL Detection (Amersham Biosciences).

Analysis of GFP expression

Live asexual blood stages and gametocytes obtained from tail blood and mature ookinetes from *in vitro* cultures were examined for GFP-fluorescence using a Leica fluorescence MDR microscope and pictures recorded using a DC500 digital camera. Hoechst-33258 staining was performed as described previously (25).

The level of GFP-expression in gametocytes was analyzed by FACS analysis. GFP-fluorescence was determined by flow cytometry using a Becton Dickinson LSR2 Benchtop Flow Cytometer (25). By gating on forward/sideward scatter, (infected) erythrocytes were selected for analysis and from each sample 30 000 cells were analyzed. Excitation was performed with a 488 nm argon-ion laser and the GFP-fluorescence was detected using a 530/30-A band pass filter. When cells were stained with Hoechst-33258, excitation was also performed with a Helium-cadmium UV laser (325 nm; UV) and the Hoechst fluorescence was detected using the 440/40-A band pass filter.

Northern blot and RNaseH analysis

RNA isolation and northern blotting was performed according to standard methods (23). RNA of *P. berghei* asexual stages, purified gametocytes or ookinetes was hybridized to probes that were generated using primers that are specific for the open reading frames of *pb28, pb25, pb000633.00.0* and *pb000245.02.0* (primer list see supplementary Table S1). As a loading control hybridization was performed with primer 87R that is specific for the 5′ end fragment of the LSU rRNA (32). For the RNaseH assay total cytoplasmic gametocyte RNA was denatured for 5 min at 70°C and hybridized for 10 min at 42°C with an oligo(dT) primer (L145B). Samples were incubated for 30 min at 37°C with RNaseH in RNase buffer (25 mM Tris–HCl, pH 8.3, 37 mM KCl, 7.1 mM $MgCl_2$, 0.5 mM dithiothreitol) and RNA fragments were detected by northern blotting and hybridization to probes of *pb28* and *pb25*.

For detection and quantification of reporter transcripts, RNA isolated from purified gametocytes of reporter parasites was hybridized to the 5′ region of *ccp2* or to the *gfp* ORF. Probes were generated by random priming and labeling with α-^{32}P-ATP using Klenow polymerase or by 5′ labeling of oligonucleotides with γ-^{32}P-ATP by phosphorylation with bacteriophage T4 polynucleotide kinase. Steady state reporter mRNA levels were determined by quantification of the hybridization signal of the *ccp2* or the *gfp* probe using the Biorad molecular imager FX and the Biorad Quantify One (version 4) software. Endogenous *ccp2* mRNA levels were used as the internal control for the total amount of female gametocyte mRNA loaded.

Bioinformatic analysis

The motif identifier program MEME (33) was used as described before (24) with the use of default settings but set to find a motif either between 40 and 50 nt or between 6 and 60 nt. The analysis was performed on the 500 bp immediately flanking upstream or downstream of a chosen ORF. Two sets of sequences were selected for analysis; those implicated and used in the physical analyses reported in this manuscript were used to identify the motif and a control group of 19 UTR regions from 10 genes selected at random from those expressed in asexual stage parasites for which sufficient sequence information was available and whose status regarding involvement in TR was unknown.

RESULTS

A GFP-reporter system to analyze the role of 3′-UTR regions in TR in gametocytes

It has previously been proposed for *p28* and *p25*, two genes encoding zygote surface proteins, that TR occurs in female gametocytes; transcripts of both genes are abundant at this stage, but translation into the protein products only occurs after formation of the gametes (21,23,34,35). These results were confirmed by northern analysis of *P. berghei* mRNA showing similar expression patterns for *pb28* and *pb25* transcripts with high levels of steady state mRNA in the gametocyte stage, whereas western analysis demonstrates that the translated products of Pb28 and Pb25 are only detectable in ookinetes and not in the gametocytes (Figure 1A). Strikingly, the sizes of the *pb28* and *pb25* transcripts appeared to be smaller in the ookinetes than in the unactivated, mature gametocytes and RNaseH analysis of gametocyte and ookinete mRNA revealed that this originates from differences in poly(A) tail length (Figure 1A). The genes *pb000633.00.0* (*pb-633-*) and *pb000245.02.0* (*pb-245-*) belong to the group of 11 genes for which TR has been implied during sexual development (24). Northern analysis confirmed the gametocyte specific transcription of both genes (Figure 1B).

A GFP-reporter system was developed that allowed the analysis of the degree of GFP expression in gametocytes of transgenic *P. berghei* parasites that contain the *gfp* ORF under control of the promoter region of the female gametocyte specific *ccp2* gene and flanked by different 3′-2UTRs (Figure 1C). First, the 3′-UTRs of the following TR-associated and gametocyte specific transcripts were analyzed: *pb28, pb25, pb-633-, pb-245-* and of *pg28*, the
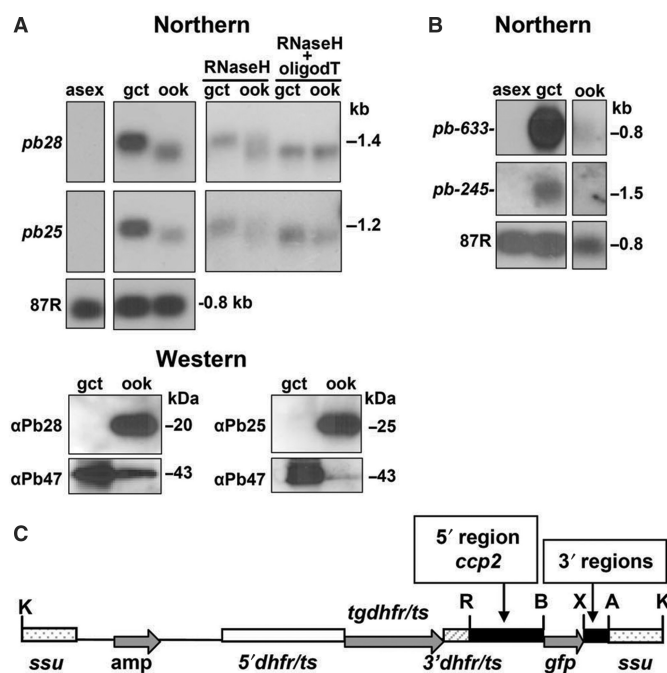
Figure 1. Model genes and reporter system to study TR in *P. berghei* gametocytes. (**A**) Expression of *P. berghei pb28* and *pb25*. Upper panel: northern analysis of mRNA of *P. berghei* asexual blood stages (asex), purified gametocytes (gct) and ookinetes (ook) hybridized with probes of the open reading frames of *pb28* and *pb25* and with the 87R probe (specific for a fragment of the LSU rRNA) as a loading control. RNase H digestion of gametocyte and ookinete RNA was performed in the absence or presence of oligo(dT). Bottom panel: western analysis of gametocyte and ookinete preparations using Pb28 and Pb25 antibodies. Antiserum against the gametocyte-specific protein Pb47 was used as a control for loading of the blot. (**B**) Northern analysis of mRNA of *P. berghei pb000633.00.0* (*pb-633-*) and *pb000245.02.0* (*pb-245-*). (**C**) Reporter constructs used to transfect *P. berghei* to generate expression of GFP under control of the female gametocyte-specific *ccp2* promoter with different 3′ regions. The constructs contain the *tgdhfr/ts* as a positive selectable marker and sequences of the *ssu* gene of the *d-rrna* locus for targeted integration into the genome. amp, ampicillin resistance gene; R, EcoRV; B, BamHI; X, XbaI; A, Asp718; K, KspI.

p28 ortholog of *pb28* in the avian malaria parasite *P. gallinaceum*. As non-repressed control 3′-UTRs (no TR) we used the 3′ regions of *P. berghei dhfr/ts*, a gene expressed in asexual blood stages (36) and the genes encoding female gametocyte specific proteins, Pb47 (van Dijk, unpublished data) and CCP2 (*pb000652.01.0*) (26). All 3′ regions used in the reporter plasmids comprised minimally the complete 3′-UTR sequence as determined by 3′ RACE of the endogenous transcript (*dhfr/ts*, *pb25*, *ccp2*, *pb47*, *pb-245-*, *pb-633-*, see Figure 2A) or from previous reports (*pb28* and *pg28*) (36–38). Reporter plasmids were transfected and after selection of stable transgenic parasite lines, correct integration of the reporter plasmids into the *small subunit* (*ssu*) of the *c/d-ribosomal-rna* locus was first established by PCR and by Southern analysis of chromosomes (data not shown). Ookinetes of all reporter parasites expressed GFP as determined by fluorescence microscopy (Figure 2C),

demonstrating that translation competent GFP transcripts were produced with all reporter constructs.

Female gametocyte specific TR is mediated by the 3′-UTR of some but not all repressed mRNAs

Figure 2A shows cartoons of the *gfp* transcripts produced by the different reporter parasite lines. Analysis of GFP protein expression showed that transcripts with the control 3′-UTR of *dhfr/ts* strongly express GFP (Figure 2B and C). Fluorescence was only observed in female gametocytes and not in asexual blood stages and male gametocytes (data not shown), confirming the female specific nature of the *ccp2* promoter. In contrast, in gametocytes of parasites expressing *gfp* transcripts with the 3′-UTR of *pb28* no GFP production was observed (Figure 2B and C). Strikingly, northern analysis revealed that transcripts containing the *pb28* 3′-UTR were in fact more abundant than those with the *dhfr/ts* 3′-UTR, suggesting TR was associated with mRNA storage (Figure 2D). Thus the *pb28* 3′-UTR is essential and sufficient to accomplish complete TR in female gametocytes of *P. berghei*. This mechanism of TR appears to be conserved in *Plasmodium* since the 3′-UTR of the *P. gallinaceum p28* efficiently inhibited GFP expression in female gametocytes of *P. berghei* as well (Figure 2B–E).

The effect on TR of five additional 3′-UTRs of genes that do (*pb25*, *pb-245-*, *pb-633-*) or do not (*pb47*, *ccp2*) undergo TR in gametocytes were analyzed. The ratio of GFP production (as measured by FACS analysis) to amount of mRNA (as measured by densitometric analysis of northern blots) was calculated for each transgenic line and normalized to values measured for control transgenic lines (Figure 2E). This revealed that only the 3′-UTR of *pb-245-* repressed translation efficiently, reducing GFP protein production relative to the steady state level of *gfp* mRNA (Figure 2C–E and supplementary Table S2). In contrast, the other four 3′-UTRs tested produced comparable GFP protein levels in female gametocytes indicating that TR of *pb25* and *pb-633-* relies on *cis*-elements located upstream of the 3′-UTR.

The sex and stage specific nature of *pb28* 3′-UTR-mediated TR was confirmed by the analysis of *gfp* expression under control of either the constitutive promoter of *eukaryotic elongation factor 1-alpha* (*ef-1αa*) (39) or the predominantly male gametocyte specific α-*tubulin II* promoter (40) from *P. berghei*. The asexual blood stages, such as trophozoites and schizonts of reporter parasites in which *gfp* was flanked by the *ef-1αa* promoter and the *pb28* 3′-UTR fluoresced strongly (Figure 3A) showing that translation of the reporter transcript was not repressed at these stages. In the exflagellating (male) gametocytes of reporter parasites expressing *gfp* under control of the α-*tubulin II* promoter and the *pb28* 3′-UTR GFP was produced at a high level (Figure 3B). Replacement of the strong α-*tubulin II* promoter with the weaker, male specific promoter of *pb000791.03.0* (26) still resulted in fluorescent male gametocytes (Figure 3B). These results demonstrate that TR mediated by the *pb28* 3′-UTR relied on transacting factors exclusive to the female gametocyte.
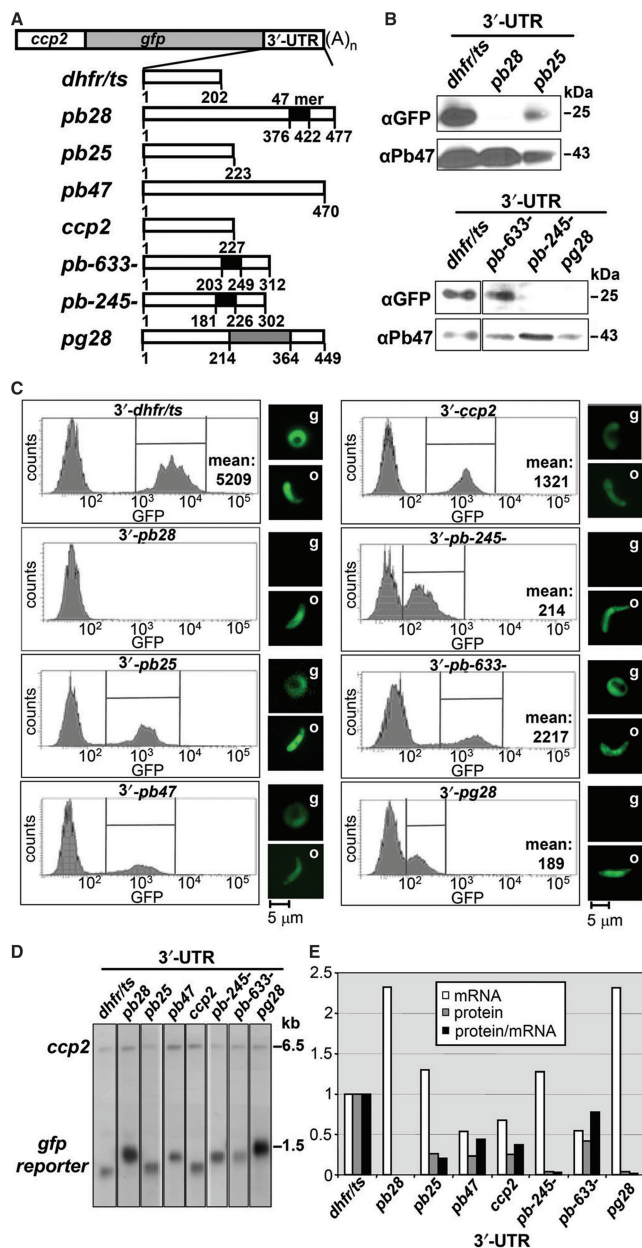
Figure 2. 3′-UTRs can mediate TR in *P. berghei* gametocytes. (**A**) Cartoons of the transcripts produced by a set of reporter constructs with the *gfp* ORF flanked by the 5′-UTR of *ccp2* and the 3′-UTR of a series of mapped gene transcripts. The predicted lengths (in nt) of the 3′-UTRs were determined by 3′ RACE on gametocyte cDNA (*dhfr/ts*, *pb25*, *pb47*, *ccp2*, *pb-245-* and *pb-633-*) or have been published before (*pb28*, *pg28*) (37,38). The positions of the predicted 47 nt TR motif (black box) or U-rich regions (grey shaded box in *pg28*) are indicated. (**B**) Western analysis of GFP production in gametocytes of transgenic *P. berghei* lines expressing *gfp* under control of different 3′-UTRs using anti-GFP antibodies. As a loading control an antiserum against the female gametocyte-specific protein Pb47 is used. (**C**) GFP fluorescence intensity in purified gametocytes of lines expressing GFP reporter transcripts with different 3′-UTRs as determined by FACS analysis of 30 000 cells. The left hand peak with fluorescent values between 0 and 10^2 are non-fluorescent cells such as male gametocytes and residual asexual stages that co-purify. The second, right hand peak represents the population of GFP-positive female gametocytes. The mean fluorescence intensity of the female gametocyte populations is indicated. Right panels: Representative GFP fluorescence images of gametocytes (g) and ookinetes (o) of the respective parasite lines. (**D**) Northern

Specific conserved regions in the pb28 3′-UTR contribute to TR in gametocytes

The functional role of the 47 nt U-rich element identified in the 3′-UTR of transcripts undergoing TR (24) was investigated. The 3′-UTR of *pb28* was divided into three regions (Figure 4A) defined as I (5′ to the 47 nt element), II (the 47 nt element) and III (3′ to the 47 nt element). Eight reporter parasite lines were tested in which *gfp* was under the control of the *ccp2* promoter but contained versions of the *pb28* 3′-UTR that either lack a region or in which regions are replaced with parts or the whole of the 3′-UTR of other genes. Deletion of region I (transcript iv) or region II (transcript i) from the *pb28* 3′-UTR resulted in a complete loss of control of TR whereas substitution of region III with the *dhfr/ts* 3′-UTR (transcript ii) had no effect on the capacity of female gametocytes to silence the GFP expression (Figure 4A–D).

The individual silencing strengths of regions I and II were assessed through the use of hybrid 3′-UTRs in which these regions were fused to the *dhfr/ts* 3′-UTR (transcripts iii and v). Both regions had the capacity to repress translation as GFP production was clearly reduced when compared with the results with the 'non-repressing' *dhfr/ts* 3′-UTR on its own (Figure 4D). These data confirm that the previously identified 47 nt motif has a strong silencing effect and show that elements in region I also contribute to TR in female gametocytes.

The role of the 47 nt motif was further investigated as before but using a variety of *pb28* 3′-UTRs with modified 47 nt motifs. Reverse complementing the 47 nt motif resulted in increased GFP expression (*i.e.* reduced TR) and was comparable to deletion of the element (see Figure 4D i, vi and supplementary Table S3). Exchange of the *pb28* 47 nt element with the putative *pb-245-* 47 nt element resulted in TR activity that was similar to the wild type *pb28* 3′-UTR (Figure 4A–D, *pb28*, vii). These results emphasize the importance of the 47 nt motif of *pb28* in TR and shows that the putative 47 nt motif of *pb-245-* is also functional in TR. However, replacement of the *pb28* 47 nt motif by the putative *pb-633-* 47 nt element did not result in TR and GFP expression in these parasites was comparable to that in parasites containing the *pb28* 3′-UTR with the reversed 47 nt motif (Figure 4A–D, vi, viii and supplementary Table S3), a result consistent with that obtained with the complete *pb-633-* 3′-UTR which did not mediate TR (Figure 2C–E).
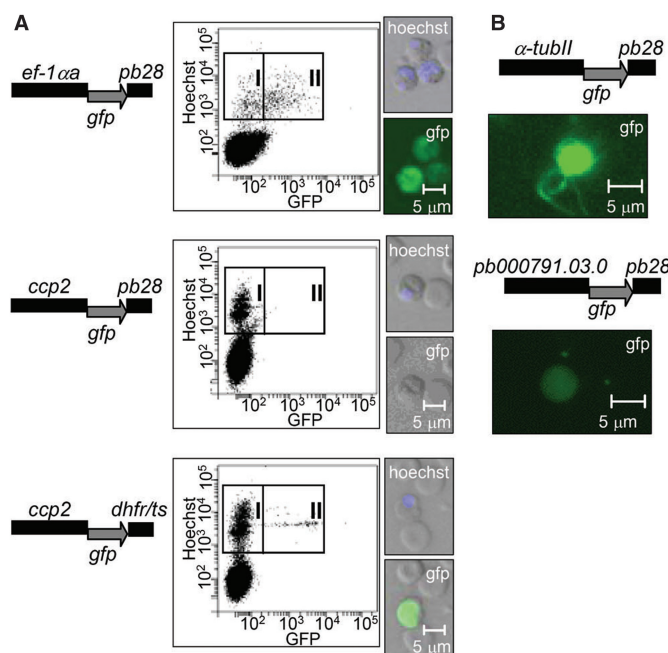
Figure 3. Factors responsible for *pb28* 3′-UTR-mediated TR are exclusively present in female gametocytes. (**A**) Each panel shows: Left, a cartoon of the transgene introduced into *P. berghei*, centre representation of the FACS analysis of Hoechst and GFP fluorescence in red blood cells infected with transgenic *P. berghei*, right, representative bright field (top) and GFP fluorescent (bottom) images of the transgenic parasites. Upper panel: Blood stage parasites expressing GFP under control of the constitutive *ef-1αa* promoter and the *pb28* 3′-UTR. Infected blood with a parasitemia of 1.5% was stained with Hoechst-33258 to be able to distinguish between infected and non-infected erythrocyte population. 57% of the Hoechst-stained cells are GFP-positive (region II) demonstrating that asexual parasites express GFP. The non-GFP fluorescent parasites (region I) are mainly young ring forms that do not express GFP (25) Middle panel: Blood stage parasites expressing GFP under control of the female specific *ccp2* promoter and the *pb28* 3′-UTR. Both asexual parasites and gameto-cytes are GFP-negative. Lower panel: Infected red blood cells expressing GFP under control of the female specific *ccp2* promoter and the *dhfr/ts* 3′-UTR. Asexual parasites are GFP-negative and only gametocytes are GFP-positive (region II). (**B**) The two panels each show: cartoons of the transgene introduced into *P. berghei* and representative GFP fluorescence images of exflagellating male gametocytes. *Gfp* is expressed under control of the *pb28* 3′-UTR and the male specific promoter regions of either *α-tubulin II* (*α-tubII*) (top) or *pb000791.03.0* (bottom) showing the lack of TR in male gametocytes.

Sequences that mediate TR exist in the 5′-UTR of pb25

The *pb25* 3′-UTR region did not exert TR, thus the possible involvement of 5′-UTRs in TR was assessed. Reporter plasmids were constructed in which *gfp* was flanked with the 3′ region of *dhfr/ts* and the 5′ region (this includes the promoter and 5′-UTR regions) of *ccp2*, *pb28* and *pb25* (Figure 5A). The 5′ regions of *pb28* and *ccp2* produced similar levels of steady state *gfp* mRNA in gametocytes of transgenic parasites but translation was much more efficient with the transcripts containing the *pb28* 5′-UTR (Figure 5B and C). The GFP protein/mRNA ratio was 14 times higher with the *pb28* 5′-UTR than with the *ccp2* 5′-UTR (Figure 5C and supplementary Table S4),

indicating the *pb28* 5′-UTR transcripts were translated more efficiently. Interestingly, parasites expressing *gfp* with the 5′ region of *pb25* produced considerably higher levels of steady state *gfp* mRNA than those with the *ccp2* or *pb28* 5′ regions (20–25 times higher, Figure 5B and C). However, despite the abundance of *gfp* transcripts produced by the *pb25* promoter, only low levels of GFP were produced (Figure 5B and C) indicating that the *pb25* 5′-UTR prevents efficient translation and thus mediates TR in gametocytes.

Placement of the ORF-proximal 350 bp of the *pb25* 5′-UTR downstream of *gfp* in an expression cassette driven by the *ccp2* promoter generated hardly any GFP production when compared with controls (*pb25* 5′-UTR in reverse orientation, Figure 5D) showing that the TR activity of the *pb25* 5′-UTR is not dependent on its position upstream of the ORF. The stability of transcripts with the *pb25* 5′-UTR was comparable to those having the *pb28* 3′-UTR and greater than that of the control transcripts (Figure 5E).

The ability of the *pb25* 5′-UTR to mediate TR prompted an analysis of this and other UTR regions used in this study for sequences with possible homology to the original 47 nt sequence element. Visual inspection, ClustalW and MEME analyses indicated that the *pb25* 5′-UTR is generally U-rich (50%) and contains U-rich regions of significant homology to the 47 nt element. In Figure 5, the U-rich region most consistently identified in the *pb25* 5′-UTR is shown. Similarly, the 3′-UTR of *pg28* has an extensive and highly U-rich region (84%) of 150 nt (indicated in Figure 2A); MEME-analysis identified homologies to the original 47 nt motif as do all regions used in this study that have been shown to mediate TR but not in control sequences taken from a random selection of asexually expressed genes (summarized in supplementary data). Based on these observations, we suggest that a U-rich region located in either the 3′- or the 5′-UTR of mRNAs subject to TR is central to the mechanism of TR in *Plasmodium* female gametocytes.

DISCUSSION

The data presented here demonstrate for the first time the involvement in TR of *cis*-acting elements residing in UTRs of affected transcripts in *Plasmodium* female gametocytes. Currently the data indicate that the RNA element mediating TR may lie in either the 5′ or more commonly the 3′ region of a given transcript in a case-specific fashion. The mechanism mediating TR through interaction with the 3′-UTR is evolutionarily conserved since the *p28* 3′-UTR from the avian parasite, *P. gallinaceum* could also mediate efficient TR in female gametocytes of the rodent parasite *P. berghei*.

The importance for TR in female gametocytes of *Plasmodium* was demonstrated here of a U-rich 47 nt element (region II in this study) that lacks simple direct repeats and was originally identified in the 3′-UTRs of the DOZI-associated transcripts subject to TR in female gametocytes (22,24). A number of features suggest that U-rich regions such as the 47 nt element are specific
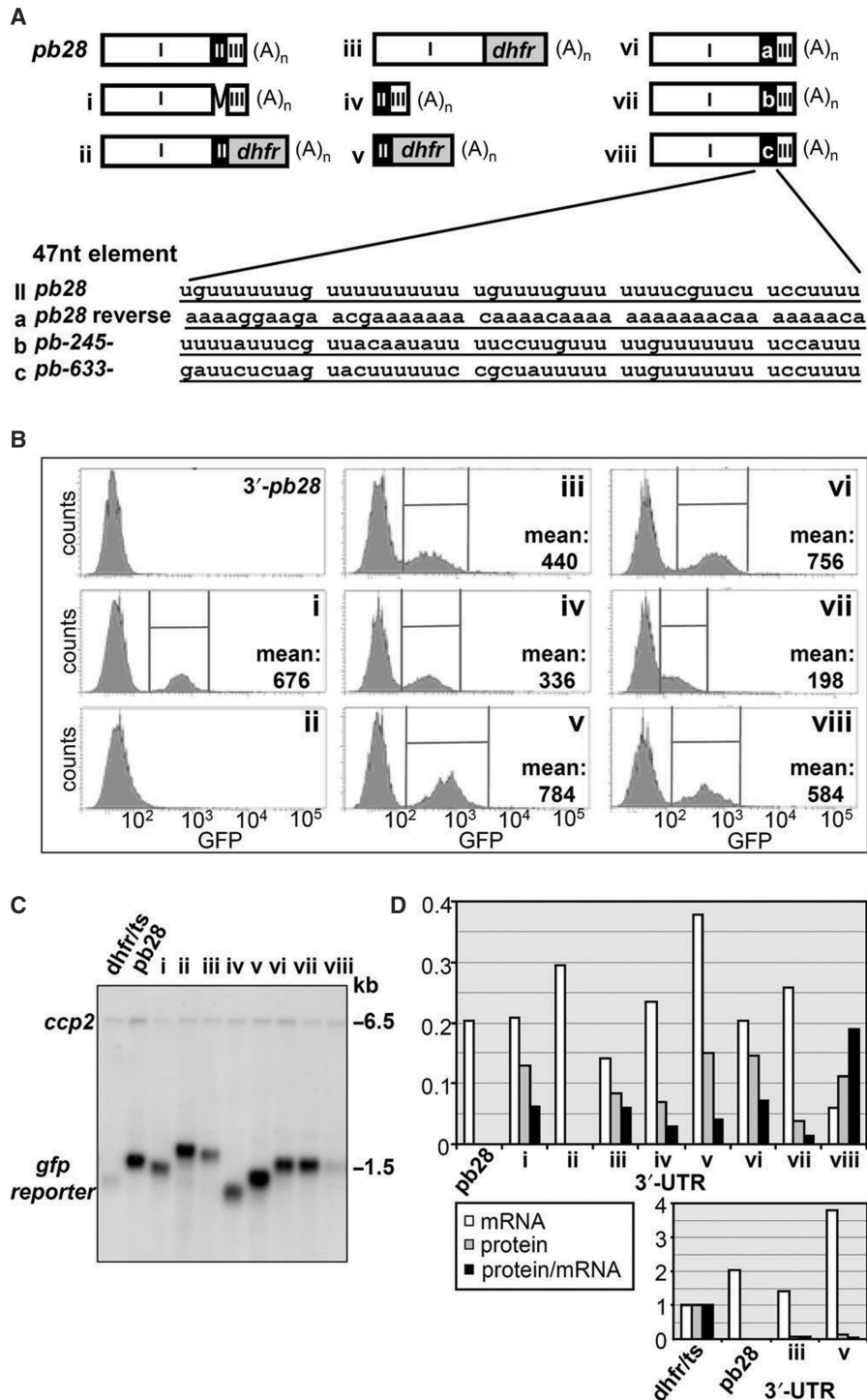
Figure 4. The 47nt 3′-UTR motif is involved in the mediation of TR. (**A**) Cartoons of the 3′-UTRs of *gfp* transcripts produced by a set of reporter constructs with *gfp* under the control of the *ccp2* promoter and mutated/truncated *pb28* 3′-UTR regions. Regions of the *pb28* 3′-UTR are defined as follows: I; 5′ part of the *pb28* 3′-UTR (bases 1–375), II; 47nt element (bases 376–422), III; 3′ part of the *pb28* 3′-UTR (bases 423–477). Fusions were made with the complete *dhfr/ts* 3′-UTR (*dhfr*). Various combinations of the four regions were tested (i–v) in addition to 3′-UTR regions in which region II of *pb28* was replaced with either the reversed sequence of the 47nt element (a) or with the putative 47nt elements of the 3′-UTR of *pb-245-* (b) and *pb-633-* (c) (vi–viii). (**B**) FACS analysis of GFP fluorescence intensity of gametocytes (as described for Figure 2C) expressing transcripts as shown in (A). (**C**) Northern analysis of steady state levels of mRNA from purified gametocytes expressing transgene transcripts as shown in (A). The *ccp2* 5′ region was used as a probe as described for Figure 2D. (**D**) Relative GFP mRNA and protein expression levels in gametocytes expressing *gfp* transcripts with 3′-UTRs as shown in (A). GFP mRNA and protein levels were determined as described for Figure 2E. Upper panel: Protein, mRNA and protein/mRNA levels displayed relative to the reporter parasite line expressing *gfp* with the *pb28* 3′-UTR. Lower panel: Protein, mRNA and protein/mRNA levels displayed relative to the *dhfr/ts* 3′-UTR control.

Figure 5. TR regulatory sequences exist in the 5′-UTR of *pb25*. (**A**) Cartoons of the GFP expression cassettes in reporter constructs for the expression of GFP under control of the promoter/5′-UTRs of *ccp2*, *pb28* and *pb25* and the 3′-UTR region of *dhfr/ts*. (**B**) GFP expression in purified gametocytes transfected with the expression cassettes as shown in (A). Upper panel: northern analysis using a *gfp* probe to detect the reporter transcripts and the female gametocyte specific *ccp2* probe as an internal control. Bottom panel: western analysis using GFP antibodies. Antiserum against the gametocyte-specific protein Pb47 was used as a control. (**C**) Relative GFP mRNA and protein expression levels in gametocytes transfected with the expression cassettes as shown in (A). GFP mRNA and protein levels were determined as described for Figure 2E. The GFP protein, mRNA and protein/mRNA level in each parasite line is presented relative to the level obtained with the control reporter strain expressing GFP under control of the *ccp2* 5′ region and the *dhfr/ts* 3′-UTR. (**D**) The two panels each show: Top, cartoons of the transgene introduced into *P. berghei* and bottom, FACS analysis of purified gametocytes transfected with reporter constructs expressing GFP under control of a 3′-UTR comprising 350 bp of the 5′-UTR of *pb25* in the right (5′pb25) and reversed (5′pb25rev) orientation. GFP fluorescence intensity of gametocytes was measured as described in Figure 2C. (**E**) Northern analysis of steady state levels of mRNA from purified gametocytes transfected with reporter constructs expressing *gfp* under control of the *ccp2* 5′ region and the 3′-UTRs of *pb28*, *ccp2* and *ccp2* with insertions of the 5′-UTR of *pb25*. The *ccp2* 5′ region was used as a probe as described for Figure 2D. (**F**) Conceptually transcribed sequence of the *pb25* upstream region that was inserted into the 3′-UTR of the reporter constructs as described in Figure 5D. The 40nt U-rich TR-associated elements identified by MEME are underlined.

mediators of TR in *Plasmodium* female gametocytes: (i) Deletion of the 47 nt element results in a loss of (tight) repression of translation, (ii) Significant TR was observed when the 47 nt element was combined with the non-repressing *dhfr/ts* 3′-UTR, (iii) The 47 nt elements of different TR-associated transcripts are interchangeable, (iv) The 5′-UTR of *pb25*, which mediates TR independent of its position in transcripts contains similar U-rich regions. Therefore, a role for the 47 nt element in mediating TR through interaction with trans-acting (protein) factors and possibly by directing the transcript to mRNPs undergoing TR can be anticipated.

In addition to the 47 nt element, the 5′ part (region I, 376 nt) of the *pb28* 3′-UTR could also mediate a degree of TR and the data indicate that both regions may act in concert to achieve complete TR. The deletion studies do not support interaction of the two regions in forming e.g. stem loop structures since each region functions independently. Although the 47 nt element of the *pb-245-* 3′-UTR was able to complement TR of the *pb28* 3′-UTR, the putative element of *pb-633-* could not, reflecting the results obtained with the complete 3′-UTRs of these genes. In the absence of an obvious structural basis for the difference in the capacity of the different 47 nt elements to mediate TR, further deletion and mutational analysis of this region and region I will be required to fully dissect the relationship between structure and function of the 3′-UTRs. The MEME analysis performed on the UTR regions used in this study identified U-rich motifs in the 5′-UTR of *pb-633-* as well (see supplementary data) raising the possibility that it is this region that mediates TR or that U-rich regions in both UTRs are necessary for TR of this

transcript. In addition, it is of interest to note that a UUGU motif is present in two copies in the 47 nt element in the 3′-UTRs of *pb28* and *pb-245-* but only once in that of *pb-633-*. In other systems this duplicated motif is known to be involved in binding to members of the Puf family of mRNA modulating (including TR) proteins (41,42). There are two homologs of Puf proteins in *Plasmodium* (PFE0935c/PB000307.01.0 and PFD0825c/PB000416.02.0) both expressed in gametocytes (43).

In addition to puf-binding elements, a number of metazoan sequence motifs have been identified in 3′-UTRs that regulate mRNA stability and translational capacity by their interaction with RNA-binding proteins (15). The release of transcripts from TR is frequently associated with an increase in the length of the poly(A) tail of the cytoplasmic mRNA and an enhanced ability to recruit poly(A) binding protein (PABP) (2). Cytoplasmic polyadenylation is dependent upon the presence of U-rich motifs termed CPE and/or eCPE that bind specific proteins such as CPEB and ElrA, respectively (15,44). All four of the *P. berghei* 3′-UTRs that were investigated and are associated with translationally repressed transcripts contain candidate CPE elements and, as noted, the 47mer is U-rich. However, our results reveal that the poly(A) tail of both *pb25* and *pb28* mRNA does not increase in length upon translation rather decreases with age of the transcript. Other well-studied 3′-UTR sequence elements involved in mRNA stability in metazoans, AREs, are rich in adenosine and/or uridine but lack a strict sequence consensus. Several families of ARE-BPs have been identified among which the ELAV/Hu RNA-binding proteins and the CELF/Bruno-like proteins (13). Two genes encoding Bruno-like proteins (PF14_0096/PB001285.00.0 and PF13_0315/PB000862.03.0) can be identified from the genomic data of *Plasmodium* and are thus candidate *trans*-acting factors for TR in female gametocytes.

We have previously shown that the protein DOZI, a DDX6 group RNA helicase, is a *trans*-acting factor essential to the process of stabilizing TR transcripts in the female gametocyte and is physically associated with the transcripts known to undergo TR and are studied here (22,24). DOZI⁻ mutant gametocytes abort development of the zygote implying that TR is central to zygote development. DDX6 helicases are highly conserved and in metazoans they play a similar gamete-associated role in TR and development of the zygote and the early embryo (45–47). However, the DDX6 helicase of *S. cerevisiae* (Dhh1p) is largely involved in a pathway of RNA degradation via cytoplasmic P-bodies (16,48) whereas it is partially located in transient RNA–protein complexes (stress granules) in Trypanosomes (17,18). TR is a dynamic process and active over at least the early stages of zygote development: P25 is produced already 30 min after activation of gamete formation, whereas P28 can only be detected at 2 h (21,23). Furthermore, transcripts of the von Willebrand factor related protein (WARP) gene are present in gametocytes and associated with DOZI whereas the protein is only detected 8 h after fertilization in the developing ookinete (22,49). Taken together these observations suggest that TR is affected through mRNP

particles of distinct protein and mRNA composition that allows a controlled timing of derepression of distinct mRNA populations during zygote development. In part the regulation of the timing of derepression may be achieved through the ability of different mRNA species to participate in distinct mRNPs determined by their UTR regions. Evidence for the temporal derepression of distinct mRNA populations and its control through specific protein interactions is accumulating in metazoans such as *Xenopus* and *Drosophila* where Musashi and members of the Puf family can play a role (50,51). Further elucidation of the role of both the 3′- and 5′-UTRs of transcripts of different genes in the timing of derepression of TR and in the exact localization of the transcripts in the cytoplasm of the female gametocyte and the developing zygote might help unravel the mechanisms of such cascades in *Plasmodium*.

Plasmodium belongs to the Alveolata distinct from the Opisthokonta, the grouping of eukaryotes that include yeast, *Drosophila*, *Caenorhabditis elegans* and man (52). The data here taken together with our previous published work (22) demonstrate that TR in female gametocytes of *Plasmodium* appears to be mediated by both *cis-* and *trans*-acting factors that are closely analogous to those employed by female germ line cells in metazoans. TR is, therefore, an ancient mechanism of post-transcriptional gene control that has evolved for use in the female germ-line cells where it is used to stabilize and store transcripts that are necessary for the early development of the post-fertilization and zygotic events. Further similarities in the components and control mechanisms of TR between *Plasmodium* and metazoans can be anticipated and *Plasmodium* may prove to be a relatively approachable system of study of TR that will provide more general insights into what must now be considered a widely conserved mechanism of post-transcriptional control of gene expression. Finally it is possible that the identification of *cis-* and *trans*-acting factors could be of value for the development of drugs or vaccines that prevent parasite transmission to the mosquito through derepression of TR in circulating gametocytes or by maintaining TR in mosquito stages. Both strategies would render the parasites incapable of transmission.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

REFERENCES

1. Vasudevan,S., Seli,E. and Steitz,J.A. (2006) Metazoan oocyte and early embryo development program: a progression through translation regulatory cascades. *Genes Dev.*, **20**, 138–146.
2. Piccioni,F., Zappavigna,V. and Verrotti,A.C. (2005) Translational regulation during oogenesis and early development: the cap-poly(A) tail relationship. *C. R. Biol.*, **328**, 863–881.
3. Colegrove-Otero,L.J., Minshall,N. and Standart,N. (2005) RNA-binding proteins in early development. *Crit Rev. Biochem. Mol. Biol.*, **40**, 21–73.
4. Allison,R., Czaplinski,K., Git,A., Adegbenro,E., Stennard,F., Houliston,E. and Standart,N. (2004) Two distinct Staufen isoforms in Xenopus are vegetally localized during oogenesis. *RNA*, **10**, 1751–1763.
5. Bashirullah,A., Cooperstock,R.L. and Lipshitz,H.D. (2001) Spatial and temporal control of RNA stability. *Proc. Natl Acad. Sci. USA*, **98**, 7025–7028.
6. Evdokimova,V., Ruzanov,P., Anglesio,M.S., Sorokin,A.V., Ovchinnikov,L.P., Buckley,J., Triche,T.J., Sonenberg,N. and Sorensen,P.H. (2006) Akt-mediated YB-1 phosphorylation activates translation of silent mRNA species. *Mol. Cell. Biol.*, **26**, 277–292.
7. Mohr,E. and Richter,D. (2001) Messenger RNA on the move: implications for cell polarity. *Int. J. Biochem. Cell Biol.*, **33**, 669–679.
8. Matova,N. and Cooley,L. (2001) Comparative aspects of animal oogenesis. *Dev. Biol.*, **231**, 291–320.
9. Wickens,M. and Goldstrohm,A. (2003) Molecular biology. A place to die, a place to sleep. *Science*, **300**, 753–755.
10. Gebauer,F. and Hentze,M.W. (2004) Molecular mechanisms of translational control. *Nat. Rev. Mol. Cell Biol.*, **5**, 827–835.
11. Mendez,R. and Richter,J.D. (2001) Translational control by CPEB: a means to the end. *Nat. Rev. Mol. Cell Biol.*, **2**, 521–529.
12. Wilkie,G.S., Dickson,K.S. and Gray,N.K. (2003) Regulation of mRNA translation by 5'- and 3'-UTR-binding factors. *Trends Biochem. Sci.*, **28**, 182–188.
13. Barreau,C., Paillard,L. and Osborne,H.B. (2005) AU-rich elements and associated factors: are there unifying principles? *Nucleic Acids Res.*, **33**, 7138–7150.
14. Espel,E. (2005) The role of the AU-rich elements of mRNAs in controlling translation. *Semin. Cell Dev. Biol.*, **16**, 59–67.
15. de Moor,C.H., Meijer,H. and Lissenden,S. (2005) Mechanisms of translational control by the 3' UTR in development and differentiation. *Semin. Cell Dev. Biol.*, **16**, 49–58.
16. Sheth,U. and Parker,R. (2003) Decapping and decay of messenger RNA occur in cytoplasmic processing bodies. *Science*, **300**, 805–808.
17. Cassola,A., De Gaudenzi,J.G. and Frasch,A.C. (2007) Recruitment of mRNAs to cytoplasmic ribonucleoprotein granules in trypanosomes. *Mol. Microbiol.*, **65**, 655–670.
18. Holetz,F.B., Correa,A., Avila,A.R., Nakamura,C.V., Krieger,M.A. and Goldenberg,S. (2007) Evidence of P-body-like structures in Trypanosoma cruzi. *Biochem. Biophys. Res. Commun.*, **356**, 1062–1067.
19. Clayton,C. and Shapira,M. (2007) Post-transcriptional regulation of gene expression in trypanosomes and leishmanias. *Mol. Biochem. Parasitol.*, **156**, 93–101.
20. Tomas,A.M., Margos,G., Dimopoulos,G., van Lin,L.H., Koning-Ward,T.F., Sinha,R., Lupetti,P., Beetsma,A.L., Rodriguez,M.C. *et al.* (2001) P25 and P28 proteins of the malaria ookinete surface have multiple and partially redundant functions. *EMBO J.*, **20**, 3975–3983.
21. del Carmen,R.M., Gerold,P., Dessens,J., Kurtenbach,K., Schwartz,R.T., Sinden,R.E. and Margos,G. (2000) Characterisation and expression of pbs25, a sexual and sporogonic stage specific protein of Plasmodium berghei. *Mol. Biochem. Parasitol.*, **110**, 147–159.
22. Mair,G.R., Braks,J.A., Garver,L.S., Wiegant,J.C., Hall,N., Dirks,R.W., Khan,S.M., Dimopoulos,G., Janse,C.J. *et al.* (2006) Regulation of sexual development of Plasmodium by translational repression. *Science*, **313**, 667–669.
23. Paton,M.G., Barker,G.C., Matsuoka,H., Ramesar,J., Janse,C.J., Waters,A.P. and Sinden,R.E. (1993) Structure and expression of a post-transcriptionally regulated malaria gene encoding a surface protein from the sexual stages of Plasmodium berghei. *Mol. Biochem. Parasitol.*, **59**, 263–275.
24. Hall,N., Karras,M., Raine,J.D., Carlton,J.M., Kooij,T.W.A., Berriman,M., Florens,L., Janssen,C.S., Pain,A. *et al.* (2005) A comprehensive survey of the Plasmodium life cycle by genomic, transcriptomic, and proteomic analyses. *Science*, **307**, 82–86.
25. Franke-Fayard,B., Trueman,H., Ramesar,J., Mendoza,J., van der Keur,M., van der Linden,R., Sinden,R.E., Waters,A.P. and Janse,C.J. (2004) A Plasmodium berghei reference line that constitutively expresses GFP at a high level throughout the complete life cycle. *Mol. Biochem. Parasitol.*, **137**, 365–367.
26. Khan,S.M., Franke-Fayard,B., Mair,G.R., Lasonder,E., Janse,C.J., Mann,M. and Waters,A.P. (2005) Proteome analysis of separated male and female gametocytes reveals novel sex-specific Plasmodium biology. *Cell*, **121**, 675–687.
27. Janse,C.J., Ramesar,J. and Waters,A.P. (2006) High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite Plasmodium berghei. *Nat. Protoc.*, **1**, 346–356.
28. de Koning-Ward,T.F., Janse,C.J. and Waters,A.P. (2000) The development of genetic tools for dissecting the biology of malaria parasites. *Annu. Rev. of Microbiol.*, **54**, 157–185.
29. Janse,C.J., Mons,B., Rouwenhorst,R.J., Van der Klooster,P.F., Overdulve,J.P. and Van der Kaay,H.J. (1985) In vitro formation of ookinetes and functional maturity of Plasmodium berghei gametocytes. *Parasitology*, **91**(Pt 1), 19–29.
30. Janse,C.J., Camargo,A., Delportillo,H.A., Herrera,S., Waters,A.P., Kumlien,S., Mons,B. and Thomas,A. (1994) Removal of leukocytes from Plasmodium vivax-infected blood. *Ann. Trop. Med. Parasitol.*, **88**, 213–216.
31. Janse,C.J. and Waters,A.P. (1995) Plasmodium-Berghei - the application of cultivation and purification techniques to molecular studies of malaria parasites. *Parasitol. Today*, **11**, 138–143.
32. van Spaendonk,R.M.L., Ramesar,J., van Wigcheren,A., Eling,W., Beetsma,A.L., Van Gemert,G.J., Hooghof,J., Janse,C.J. and Waters,A.P. (2001) Functional equivalence of structurally distinct ribosomes in the malaria parasite, Plasmodium berghei. *J. Biol. Chem.*, **276**, 22638–22647.
33. Bailey,T.L. and Gribskov,M. (1998) Methods and statistics for combining motif match scores. *J. Comput. Biol.*, **5**, 211–221.
34. Thompson,J. and Sinden,R.E. (1994) In situ detection of Pbs21 mRNA during sexual development of Plasmodium berghei. *Mol. Biochem. Parasitol.*, **68**, 189–196.
35. Vervenne,R.A., Dirks,R.W., Ramesar,J., Waters,A.P. and Janse,C.J. (1994) Differential expression in blood stages of the gene coding for the 21-kilodalton surface protein of ookinetes of Plasmodium berghei as detected by RNA in situ hybridisation. *Mol. Biochem. Parasitol.*, **68**, 259–266.
36. vanDijk,M.R., Vinkenoog,R., Ramesar,J., Vervenne,R.A.W., Waters,A.P. and Janse,C.J. (1997) Replication, expression and segregation of plasmid-borne DNA in genetically transformed malaria parasites. *Mol. Bioch. Parasitol.*, **86**, 155–162.
37. Golightly,L.M., Mbacham,W., Daily,J. and Wirth,D.F. (2000) 3' UTR elements enhance expression of Pgs28, an ookinete protein of Plasmodium gallinaceum. *Mol. Biochem. Parasitol.*, **105**, 61–70.
38. Margos,G., van Dijk,M.R., Ramesar,J., Janse,C.J., Waters,A.P. and Sinden,R.E. (1998) Transgenic expression of a mosquito-stage malarial protein, Pbs21, in blood stages of transformed Plasmodium berghei and induction of an immune response upon infection. *Infect. Immun.*, **66**, 3884–3891.
39. de Koning-Ward,T.F., Speranca,M.A., Waters,A.P. and Janse,C.J. (1999) Analysis of stage specificity of promoters in Plasmodium berghei using luciferase as a reporter. *Mol. Biochem. Parasitol.*, **100**, 141–146.
40. Kooij,T.W., Franke-Fayard,B., Renz,J., Kroeze,H., van Dooren,M.W., Ramesar,J., Augustijn,K.D., Janse,C.J. and Waters,A.P. (2005) Plasmodium berghei alpha-tubulin II: a role in both male gamete formation and asexual blood stages. *Mol. Biochem. Parasitol.*, **144**, 16–26.
41. Tadauchi,T., Matsumoto,K., Herskowitz,I. and Irie,K. (2001) Post-transcriptional regulation through the HO 3'-UTR by Mpt5, a yeast homolog of Pumilio and FBF. *EMBO J.*, **20**, 552–561.

42. Wickens,M., Bernstein,D.S., Kimble,J. and Parker,R. (2002) A PUF family portrait: 3'UTR regulation as a way of life. *Trends Genet.*, **18**, 150–157.

43. Cui,L., Fan,Q. and Li,J. (2002) The malaria parasite Plasmodium falciparum encodes members of the Puf RNA-binding protein family with conserved RNA binding activity. *Nucleic Acids Res.*, **30**, 4607–4617.

44. Wu,L., Good,P.J. and Richter,J.D. (1997) The 36-kilodalton embryonic-type cytoplasmic polyadenylation element-binding protein in Xenopus laevis is ElrA, a member of the ELAV family of RNA-binding proteins. *Mol. Cell Biol.*, **17**, 6402–6409.

45. Ladomery,M., Wade,E. and Sommerville,J. (1997) Xp54, the Xenopus homologue of human RNA helicase p54, is an integral component of stored mRNP particles in oocytes. *Nucleic Acids Res.*, **25**, 965–973.

46. Minshall,N., Thom,G. and Standart,N. (2001) A conserved role of a DEAD box helicase in mRNA masking. *RNA*, **7**, 1728–1742.

47. Nakamura,A., Amikura,R., Hanyu,K. and Kobayashi,S. (2001) Me31B silences translation of oocyte-localizing RNAs through the formation of cytoplasmic RNP complex during Drosophila oogenesis. *Development*, **128**, 3233–3242.

48. Coller,J.M., Tucker,M., Sheth,U., Valencia-Sanchez,M.A. and Parker,R. (2001) The DEAD box helicase, Dhh1p, functions in mRNA decapping and interacts with both the decapping and deadenylase complexes. *RNA*, **7**, 1717–1727.

49. Yuda,M., Yano,K., Tsuboi,T., Torii,M. and Chinzei,Y. (2001) von Willebrand factor A domain-related protein, a novel microneme protein of the malaria ookinete highly conserved throughout Plasmodium parasites. *Mol. Biochem. Parasitol.*, **116**, 65–72.

50. Charlesworth,A., Wilczynska,A., Thampi,P., Cox,L.L. and MacNicol,A.M. (2006) Musashi regulates the temporal order of mRNA translation during Xenopus oocyte maturation. *EMBO J.*, **25**, 2792–2801.

51. Wharton,R.P. and Aggarwal,A.K. (2006) mRNA regulation by Puf domain proteins. *Sci. STKE*, **2006**, e37.

52. Baldauf,S.L. (2003) The deep roots of eukaryotes. *Science*, **300**, 1703–1706.