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A conserved U-rich RNA region implicated in regulation of translation in \textit{Plasmodium} female gametocytes

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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, \textit{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 \textit{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 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 (10–12). 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 (13).

The significance and mechanisms of TR in the control of gene expression during development of unicellular eukaryotes are relatively less well-studied. \textit{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 \textit{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 develop into a single cell organism to the mosquito vector. Gametocytes are abundantly expressed on the surface of the ookinete and play a critical role 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 \textit{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 \textit{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 \textit{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 mRNAs (22). The data suggest that TR is a central mechanism for regulation of gene expression during sexual development of \textit{Plasmodium} and shares features with TR in metazoans. A 47 nucleotide (nt) U-rich cis-acting motif was identified in the 3′-UTRs of a subset of translationally repressed transcripts in \textit{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 \textit{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 pb0000245.02.0 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 \textit{Plasmodium}.

MATERIAL AND METHODS

GFP reporter constructs

The GFP expression plasmid PbGFP\textsubscript{CON} (25) was used to construct a series of \textit{P. berghei} reporter plasmids that direct expression of \textit{gfp} under control of different \textit{Plasmodium} promoter/5′-UTRs and 3′-UTRs. The \textit{ef-1aa} 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 \textit{P. berghei} reference clone 1c5y1 and \textit{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 (cep2), pb000609.00.00 (\textit{a-tubulin II}) and pb000791.03.00 have been described (26).

Transfection and genotype analysis of transgenic parasites

Transfection of \textit{P. berghei} parasites (reference wild type parasites of c15cy1, 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 \textit{P. berghei} generating transgenic lines as listed in supplementary Tables S2–S4. Correct integration of the constructs into the \textit{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 \textit{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 NaHCO\textsubscript{3}, 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 Tsuibo), 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 ookinete 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

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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 6 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 RNome buffer (25 mM Tris–HCl, pH 8.3, 37 mM KCl, 7.1 mM MgCl2, 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-32P-ATP using Klenow polymerase or by 5' labeling of oligonucleotides with γ-32P-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 pg28 and pg25, 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 (Figure 1A). Strikingly, the sizes of the pb28 and pb25 transcripts appeared to be smaller in the ookinetes than in the unactivated, mature gametocytes (24).

Northern analysis

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
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-1aa)* (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-1aa* 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.
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). 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, vii). These results were consistent with the obtained with the complete pb-633-3′-UTR which did not mediate TR (Figure 2C–E).

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, pb28, 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 transgenic 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 102 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 analysis of steady state levels of mRNA from purified transgenic gametocytes expressing gfp transcripts as shown in (A). The ccp2 5′ region was used as a probe which hybridizes to the gfp reporter transcripts as well as to endogenous ccp2 mRNA that serves as a loading control. (E) Relative GFP mRNA and protein expression levels in gametocytes expressing gfp transcripts as shown in (A). The GFP protein production is defined as the mean GFP-fluorescence intensity as determined by FACS analysis. GFP mRNA production of the same samples is determined by quantification of the intensity of the hybridization signal on northern blots relative to the internal control (ccp2). The GFP protein, mRNA and protein/mRNA level in each parasite line is normalized relative to the level obtained with the control reporter strain expressing GFP under control of the dhfr/ts 3′-UTR.
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 5′- 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

**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/tis 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 pb25 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.

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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.
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 ph28 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 (PF0046325c/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 CPE 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 ph25 and ph28 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.

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