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A molecular switch in the efficiency of translation reinitiation controls expression of *var2csa*, a gene implicated in pregnancy associated malaria

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Summary

Plasmodium falciparum malaria parasites export the protein PfEMP1 to the surface of infected erythrocytes, enabling them to adhere to receptors in the microvasculature and thereby avoid clearance by the spleen. The gene *var2csa* encodes the form of PfEMP1 that binds specifically within the placenta, causing pregnancy associated malaria, and appears to not be expressed in the absence of a placenta. We previously described an upstream open reading frame (uORF) that is responsible for repression of translation of the downstream ORF (dORF) that encodes VAR2CSA, thus keeping the gene silent when parasites infect non-pregnant individuals. To elucidate the molecular mechanism by which this repression is overcome during pregnancy, we stably transformed parasites with reporter gene constructs designed to detect switches in the efficiency of dORF translation. We found that proper regulation of switching relies on two separate components, i) active translation of the uORF and ii) sequence-specific characteristics of the surrounding transcript, which together control the ability of the ribosome complex to reinitiate a second round of translation and thus express VAR2CSA. These results provide the first details of a molecular switch that allows parasites take advantage of the unique niche provided by the placenta.

Keywords

PfEMP1; virulence; *Plasmodium falciparum*; antigenic variation; cytoadhesion; upstream ORF

INTRODUCTION

Plasmodium falciparum infected red blood cells (iRBCs) have the ability to bind to different host endothelial surface receptors, a process known as cytoadherence (Kyes, Horrocks, and Newbold, 2001). This property can disrupt blood flow to the surrounding tissue and is responsible for many of the severe complications of malaria including cerebral malaria, renal failure and pulmonary edema. Similarly, if iRBCs bind within the vasculature of the placenta they can cause pregnancy associated malaria (PAM), a syndrome that poses severe consequences for both the mother and the developing fetus (Rogerson, Mwapasa, and Meshnick, 2007). The adhesive molecule responsible for cytoadhesion is *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), a protein expressed by the parasite and exported to the iRBC surface. Parasites are capable of expressing different forms of PfEMP1 that bind specifically to different host receptors (for example CD36, ICAM1, CSA, etc.) (Kraemer and Smith, 2006). Each PfEMP1 variant is encoded by a different gene within the large,

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multicopy *var* gene family (Su *et al.*, 1995). There are approximately 60 *var* genes in each parasite genome and only one is actively transcribed at a time while the others are kept silent through a process called mutually exclusive expression (Scherf *et al.*, 1998). Hence, a population of parasites only transcribes one *var* gene at a time, thereby avoiding the display of other PfEMP1 variants to the host immune system. By switching expression to different forms of PfEMP1 relatively infrequently, the parasite is able to efficiently utilize its large repertoire of PfEMP1 variants via a process called antigenic variation, allowing them to maintain a chronic infection (Smith *et al.*, 1995). The primary level of control of *var* gene expression and antigenic variation is thought to be at the level of transcription initiation, controlled epigenetically through histone modifications.

A single form of PfEMP1 has been shown to enable iRBCs to adhere specifically within the placenta (Hviid and Salanti, 2007). This PfEMP1, called VAR2CSA, recognizes the proteoglycan chondroitin sulfate A (CSA), a molecule that is highly expressed on the surface of the syncytiotrophoblasts of the placenta (Salanti *et al.*, 2004; Viebig *et al.*, 2005). This protein is encoded by *var2csa*, a gene present within the genomes of the majority if not all parasite field isolates (Trimnell *et al.*, 2006). The ubiquitous presence of this particular *var* gene suggests that there is a strong selective pressure for parasites to maintain this conserved gene, perhaps to take advantage of the unique niche provided by the placenta. The binding of the iRBCs within the placenta causes PAM, a syndrome that includes maternal anemia, low birth weight newborns as well as high occurrence of perinatal and infant mortality (Rogerson, Mwapasa, and Meshnick, 2007).

In geographical regions endemic for malaria, people develop an antibody response against different forms of PfEMP1 after multiple infections, leading to the acquisition of age dependent immunity (Giha *et al.*, 2000). This results in the development in most individuals of a semi-immune status that prevents severe complications of the disease, but that doesn't completely eliminate parasitemia. However, even after frequent infections and exposure to most PfEMP1 forms, women in their first pregnancy are vulnerable to PAM (McGregor, 1984; Mutabingwa, 1994). This appears to result from a lack of antibodies that recognize VAR2CSA, the form of PfEMP1 associated with cytoadhesion within the placenta. However, women who have experienced PAM are resistant during subsequent pregnancies, coinciding with the development of antibodies specific to VAR2CSA (Staalsoe *et al.*, 2004). Thus parasites that express VAR2CSA are only detectable in infected pregnant women, raising the possibility that expression of the gene encoding this protein is repressed when parasites infect non-pregnant individuals. Consistent with this hypothesis, antibodies against VAR2CSA are only detected in women who have suffered from PAM but not in children, men or women who have not been pregnant (Fried *et al.*, 1998; Beeson *et al.*, 1999; Maubert *et al.*, 1999) either because parasites do not express VAR2CSA in the absence of a placenta, or alternatively because such parasites fail to cytoadhere and are cleared from the circulation before a robust antibody response is generated. However, it has been described that *var2csa* mRNA is detectable in parasites infecting non-pregnant individuals, but that the encoded protein cannot be detected (Lavstsen *et al.*, 2005; Duffy *et al.*, 2006). This raises the possibility that in addition to the transcriptional regulation that applies to all members of the *var* gene family, expression of VAR2CSA is also regulated at the level of protein translation, and that translation might be suppressed when parasites infect non-pregnant individuals. This makes *var2csa* unique among *var* genes in that it is regulated both at the transcriptional and translational levels (Amulic *et al.*, 2009; Mok *et al.*, 2008).

var2csa has a unique 5′ upstream sequence (UpsE) that is different from all other *var* genes (Lavstsen *et al.*, 2003). It contains an upstream open reading frame (uORF) of 360 bp that is situated between the transcription start site and the downstream open reading frame (dORF) that encodes VAR2CSA. This results in a bicistronic mRNA in which the two ORFs are

separated by an intercistronic region (ICR) of 269 nt. Analysis of transcripts obtained from both cultured parasites selected for binding to CSA (Lavstsen *et al.*, 2003) and transgenic parasites containing the *var2csa* upstream regulatory domain (Amulic *et al.*, 2009) confirmed that the uORF is not spliced from the message and that no alternative transcription start site exists that would result in a transcript that excludes the uORF. Thus it appears that the VAR2CSA mRNA includes both ORFs. This bicistronic structure is unique among *var* genes and is conserved in *var2csa* from all parasite isolates. Previous results from our group showed that the uORF plays a regulatory role and is responsible for repression of VAR2CSA translation (Amulic *et al.*, 2009). We proposed that the uORF represses VAR2CSA translation when parasites infect individuals that are not pregnant. However, how this repression is relieved in the presence of a placenta is not understood. Our current work is directed toward elucidating the mechanism by which the influence of the uORF is overcome and the dORF encoding VAR2CSA is efficiently translated, thereby enabling parasites to bind efficiently within a placenta.

Translation of proteins is a key process in living cells and translation initiation is a prominent point of regulation (Pain, 1996; Kozak, 1999; Valasek, 2012). This process begins with the recruitment of the small subunit of the ribosome (40S) and translation initiation factors (eIFs) to the m7G cap structure of the mRNA. This initiates the movement of the ribosome along the mRNA as it scans the sequence until a start codon (AUG) in a favorable context is recognized. The large ribosomal subunit (60S) and other initiation factors (forming the eIF2-Met-tRNAi ternary complex) then join to start the elongation step. It is thought that during elongation the majority of initiation factors are released and when the complex reaches a termination codon, both ribosomal subunits dissociate and are released from the mRNA. In the case of a bicistronic mRNA (Figure 1A), the ribosome will usually recognize and translate the uORF and once it reaches the stop codon, it will dissociate, thus preventing the dORF from being translated into protein (Figure 1B). This mechanism results in the uORF being preferentially translated due to its proximity near the cap structure. Only under certain conditions is the dORF efficiently translated (Kozak, 2002). In the case of *var2csa*, we hypothesize that when the gene is transcribed in the absence of a placenta, the uORF is translated, thus leaving the PfEMP1 encoding dORF silent. This prevents premature exposure of VAR2CSA to the immune system of women when they are not pregnant, and thereby preserves the usefulness of this form of PfEMP1. However when infecting a pregnant woman, a population of parasites arises which have overcome the repressive effect of the uORF and is able to translate the dORF.

The molecular mechanism that promotes VAR2CSA translation in the presence of a placenta is unknown, however several regulatory mechanisms that allow dORF translation in bicistronic mRNAs have been described in model organisms (Kozak, 2002; Sonenberg and Hinnebusch, 2009; Firth and Brierley, 2012). These include initiation codon “skipping”, internal ribosomal initiation and translation reinitiation. Initiation codon “skipping” is a mechanism commonly used by viruses and mammals, but rarely observed in yeast (Kozak, 2002). It describes situations in which the ribosome can pass over the start codon of a uORF and recognize a downstream start codon in a more optimal context, thereby allowing dORF translation (Figure 1C). Internal ribosomal entry sites (IRESs) are sequences within the ICR that fold into a structure that resembles the m7G cap that ribosomes typically bind to prior to scanning (Plank and Kieft, 2012). This enables ribosomes to begin scanning downstream of the start codon of the uORF, thus leading to efficient initiation at the start codon of the dORF (Figure 1D). Reinitiation of translation represents a third method for overcoming the repressive effect of a uORF. In these instances, after first translating the uORF, the ribosome retains the initiation factors or is “recharged” with them and initiates translation a second time at the start codon of the dORF (Figure 1E) (Jackson, Hellen, and Pestova, 2012). In systems where it has been studied, the efficiency of dORF translation is typically regulated

by post-translation modification events (typically phosphorylation) of various ribosomal initiation factors (Hinnebusch, 2005; Valasek, 2012). By understanding the molecular basis for overcoming the repressive effect of a uORF, it has been possible to decipher how different organisms sense changes in their environment and respond accordingly (Morris and Geballe, 2000).

To determine how malaria parasites control VAR2CSA expression, we utilized reporter gene constructs designed to determine which mechanism is employed by parasites when they switch from the repressed to the highly expressed state of VAR2CSA translation. Our results excluded both the initiation codon "skipping" and internal initiation mechanisms, while a reinitiation mechanism was consistent with all of the observed results. Further, the efficiency of reinitiation was highly sensitive to changes in both the length of the uORF and the sequence of the upstream region of the transcript. The data suggest a two component switching mechanism that regulates the efficiency of reinitation by altering the retention of initiation factors by the ribosome as it translates the uORF. Using parasites co-transformed with two independent constructs, we found evidence that transcripts can compete for limiting transacting factors that influence the rate of reinitiation. These experiments suggest that when infecting non-pregnant individuals, VAR2CSA expression is repressed due to inefficient translation reinitiation at the start codon of the PfEMP1 encoding dORF. However, in the presence of a placenta, a population of parasites arises in which the translating ribosome retains translation initiation factors beyond the uORF stop codon, in turn leading to an increase in the efficiency of reinitiation. This allows for substantially increased expression of VAR2CSA and enables parasites to adhere to CSA and take advantage of the new environmental niche provided by the placenta.

RESULTS

Verification of the *var2csa* uORF as a translational repressor

Experimental design—It was previously shown that *var2csa* is transcribed into a bicistronic mRNA that conforms to the basic structure shown in Figure 1A (Lavstsen *et al.*, 2003). In this case, the dORF encodes the chondroitin sulfate binding form of PfEMP1 known as VAR2CSA that is linked to PAM. Our previous work demonstrated that translation of the uORF represses translation of the dORF (Amulic *et al.*, 2009), consistent with the model shown in Figure 1B. The current investigation exploited our previous experimental design in which parasites were stably transfected with plasmid constructs containing the *var2csa* upstream regulatory region (UpsE) driving expression of the *blasticidin S deaminase* (*bsd*) selectable marker. In these experiments, the *var2csa* promoter has been separated from the regulatory element found in *var* introns, thus it is always transcriptionally active (Deitsch, Calderwood, and Wellems, 2001). Therefore changes in uORF or dORF expression can be attributed to changes in translational efficiency and not to the types of transcriptional regulation that *var* genes are known for. The original construct (V2B, Figure 2B) includes the UpsE transcription start site and regulatory region, the intact uORF and the ICR fused to the *bsd* gene in place of the dORF. Due to its position as the dORF in the bicistronic transcript, the *bsd* open reading frame is not efficiently translated and the transfected parasites are sensitive to blasticidin. However, when parasites are cultured in the presence of blasticidin, it is possible to select parasites that have switched to efficient translation of the dORF and thereby become resistant to the drug. This provides an experimental system that enables us to easily obtain parasites in which translation of the dORF was either active or repressed, and thereby to investigate the details of this regulatory pathway. Our earlier work described the characteristics of the repressive effect of the uORF (Amulic *et al.*, 2009). Here we extend this work to investigate how parasites overcome this repression and switch to efficient translation of the dORF.

Baseline uORF repression—When a culture was seeded with V2B transfected parasites at an initial parasitemia of 0.075%, in the absence of blasticidin, a parasitemia of 5% was reached in 4 days (Figure 2B). A parallel culture grown under blasticidin pressure showed an initial growth delay and took an additional 8 days to reach the same parasitemia. Once established however, the blasticidin resistant parasites grew robustly, suggesting that a small subpopulation of parasites that were resistant to blasticidin was selected, and that these parasites were able to efficiently translate the dORF. While measuring the number of days required for parasites to reach 5% parasitemia is a relatively coarse measurement, this assay is highly reproducible, consistently yielding identical numbers in several independent selection experiments, thus providing us with a useful tool to assay for dORF translational efficiency. The levels of mRNA were similar under both growth conditions (Figure 2B), providing additional evidence that blasticidin expression is repressed at the translational level in the unselected culture. Assays were also performed with a similar construct (V2mB, Figure 2C) that has a single base pair mutation in the uORF start codon that abolishes it as a translation start site, thus preventing uORF translation. Parasites carrying this construct grew at the same rate with and without blasticidin, thus demonstrating that translational repression of the dORF is dependent on translation of the uORF. This also indicates that any additional ORFs of shorter length that occur by chance within the leader sequence of the transcript appear not to affect translation of the dORF, since expression of the dORF is fully rescued by disruption of the initiation codon of the uORF (Figure 2C and (Amulic *et al.*, 2009). Lastly, to ensure that the addition of an HpaI restriction site inserted near the initiation codon of the dORF in our constructs did not affect translational efficiency, the wildtype sequence was restored using site directed mutagenesis. Parasites transfected with the resulting construct behaved similarly to those transfected with V2B (Figure 2D), indicating that the presence of the HpaI site did not change translation initiation at the dORF. These data are consistent with our previously published results (Amulic *et al.*, 2009) and establish an empirical method for measuring the efficiency of translation of the dORF.

Basic mechanism for overcoming translational repression: “skipping” vs IRES vs reinitiation

Initiation codon “skipping” is not responsible for dORF translation—As shown in Figure 1, several mechanisms have been established in model organisms for overcoming the translational repression of an uORF. To determine if one of these mechanisms is responsible for efficient translation of the dORF in the *var2csa* transcript, we undertook a systematic analysis to test all three established mechanisms. The first mechanism tested was initiation codon “skipping” (or “leaky scanning”) in which the scanning ribosome bypasses the start codon of the uORF and instead initiates translation at the start codon of the dORF (Figure 1C). This mechanism typically relies on the trait that the start codon of dORF is within a more optimal sequence context than that of the uORF, thus if AUG recognition by the scanning ribosome becomes more stringent, the start codon of the uORF can be skipped, leaving the uORF untranslated and allowing the ribosome to continue scanning and move efficiently to the start codon of the dORF (Kozak, 2002). The V2cB construct was made replacing the 10 bp upstream of the start codon of the uORF with the sequence found immediately upstream of the dORF start codon (Figure 3A). If this sequence represents a more optimal context for translation initiation, it should not be skipped by the ribosome, even if start codon initiation has become more stringent, and thus the transgenic parasites should not be able to efficiently skip translation of the uORF and express the *bsd* ORF. However, under blasticidin selection, parasites were readily able to translate *bsd* and survive. In fact, they took fewer days to re-establish a high parasitemia, suggesting that differences in the efficiencies of the start codons of the two ORFs was not a major contributor to the switching mechanism, thus failing to support the “skipping” mechanism.

A second experiment was performed to test the "skipping" mechanism. With the "skipping" model, a scanning ribosome translates either the uORF or the dORF, but not both (Figure 1C). Therefore, increased translation of the dORF should result in a concomitant decrease in uORF translation, and thus less expression of the protein encoded by the uORF. To test this model, we replaced uORF element in our constructs with the coding regions of the reporter genes *Gaussia* and *Renilla luciferases* (V2GB and V2RB constructs, Figures 3B and C, respectively) and measured the luminescence levels of the transgenic parasites cultured with and without blasticidin pressure. We also made an additional construct (V2cGB, Figure 3D), a derivative of V2cB, where the uORF was replaced by *Gaussia luciferase*. These parasite lines displayed an increase in the amount of time needed for blasticidin resistance to be selected (a property that will be considered in detail below), however mRNA levels did not change and luminescence levels did not decrease when parasites switched to efficient translation of the dORF (under blasticidin pressure) (Figure 3B-D, right). Thus translation of the uORF remained unchanged and it appears that the selected parasites are translating both ORFs simultaneously. Both assays provide evidence inconsistent with a model of initiation codon "skipping" for the dORF translation of *var2csa*.

Internal initiation mechanism (IRES) is not responsible for dORF translation— Ribosomes typically require the 5′ m7G cap for mRNA recognition (Kozak, 1999). However, internal initiation mechanisms have been observed in some viruses, where ribosomes can recognize an internal ribosomal entry site (IRES) placed in the middle of the transcript (Figure 1D) (Cullen, 2009). To determine if this mechanism might apply to *var2csa* translation, we changed the sequence of the *var2csa* segments that might contain an IRES: the uORF and ICR elements (V2dsB, Figure 3E). The uORF sequence was replaced with a sequence of identical length and that encoded the same amino acids, but that used alternative codons and thus was highly divergent. The ICR sequence was "scrambled", but the overall AT content was conserved. Parasites transfected with V2dsB readily translated the dORF and survived blasticidin selection (Figure 3E) suggesting the nucleotide sequence of the uORF and ICR are not required for translation of the dORF, data inconsistent with the presence of IRES element.

Reinitiation results in dORF translation—Translation reinitiation is characterized by the ability of the ribosome to restart translation after having completed translation of the uORF (Figure 1E). Thus, two different proteins can be translated from a single bicistronic transcript. The luciferase expressing constructs (Figures 3B, C and D) provide data consistent with this model. Reinitiation depends on retention of certain initiation factors by the ribosome complex until it reaches the start codon of the dORF, thus enabling it to reassemble a complex capable of initiating a second round of translation. Initiation factors can remain transiently associated with the elongating ribosome, therefore increasing the uORF length or the ribosome transit time through the uORF increases the likelihood that the initiation factors are released prior to reaching the start codon of the dORF, thus reducing the efficiency of reinitiation (Kozak, 2001). The length of the uORF therefore can have a profound effect on the efficiency of reinitiation. In our previous work with the *var2csa* upstream region, we found that significantly shortening the uORF length through the introduction of a premature stop codon greatly reduced repression of dORF translation, an observation consistent with this model (Amulic *et al.*, 2009). Using the current approach, we found that constructs with significantly longer uORFs (V2GB and V2RB, Figures 3B and C, respectively) greatly increased the number of days required to select a blasticidin resistance population of parasites as predicted, providing further evidence in support of a reinitiation mechanism for translation of the dORF in the *var2csa* transcript.

Cis features of the *var2csa* reinitiation mechanism

ICR sequence is important for reinitiation efficiency—The length of the uORF has a significant effect on reinitiation efficiency. It has also been described that the length of the ICR can be important, since a longer ICR can facilitate dissociation of initiation factors or, alternatively it may allow the ribosome an extended period to "recharge" with initiation factors (Kozak, 1987). To determine if the length of the ICR affects the efficiency of reinitiation, we altered the length of this sequence to 0.4, 1.5 and 2 times the length of the region found in the endogenous *var2csa* transcript (V2B0.4×5′ICR, V2B1.5×ICR and V2B2×ICR; Figures 3F-H, respectively). Surprisingly, in all cases the efficiency of reinitiation was greatly increased, reducing the time needed to select a blasticidin resistant population to a single day, demonstrating that the repressive effect of the uORF was nearly eliminated. These data, while not eliminating the possibility that ICR length can influence reinitiation frequency, suggest that the sequence of the ICR plays a more prominent role in enabling the uORF to suppress translation reinitiation and expression of the dORF.

To further investigate the role of the ICR sequence, additional constructs were prepared in which this element was more precisely modified. In V2BsICR (Figure 3I) the ICR was replaced by a sequence of identical size and AT content, but with a scrambled sequence. V2B0.6×3′ICR (Figure 4A) contains only the 3′ 0.6 of the ICR, and in V2BinvICR (Figure 4B) the ICR was inverted relative to its orientation in the *var2csa* transcript. Similar to the changes in length, all of these constructs displayed greatly diminished repression of dORF expression, nearly eliminating the time needed to select a blasticidin resistant population.

The importance of the ICR sequence could be do to a specific secondary structure that forms within this region of the transcript. Computational programs (KineFold, MFold) designed to predict mRNA secondary structure were used to identify potential structures that might form within the ICR, and both identified the presence of a putative hairpin near the 3′ end of the ICR (nt 169-249). Disruption of this predicted hairpin through the introduction of 9 point mutations at critical sites (V2BunstICR, Figure 4C and supplemental Figure 1) did result in greatly reduced dORF translational repression, however attempts to rescue repression by introducing compensatory mutations that were predicted to re-establish the putative hairpin (Figure V2BstICR, Figure 4D and supplemental Figure 1) failed. Nonetheless, it is clear that translational repression of the dORF is highly dependent on the ICR sequence.

As expected, complete elimination of the ICR resulted in very little translational repression (V2BØICR, Figure 4E). Interestingly however, increasing the length of the uORF was able to rescue translational repression even in the absence of the ICR (V2GBØICR and V2RBØICR, Figures 4F and 4G), although not to the same extent as observed with an intact ICR (compare to V2GB and V2RB, Figures 3B and 3C). Thus the length of the uORF and the sequence of the ICR both appear to independently contribute to repression of translation of the dORF.

Effect of uORF and 5′ UTR sequences on repression—Given the importance of the ICR sequence for repression of translation of the dORF, we chose to investigate if similar sequence requirements were found within regions of the transcript upstream of the ICR. First, the uORF sequence was replaced with a sequence of identical length and that encoded the same amino acids, but that used alternative codons and thus was highly divergent (V2BduORF, Figure 4H). This construct is similar to V2dsB (Figure 3E) except the sequence of the ICR was not altered. In parasites transfected with this construct translational repression was disrupted and blasticidin resistant parasites were obtained after a single day of selection. This construct shows that repression is highly sensitive to changes in sequence of the uORF.

To determine if the sequence requirements for translational repression extend into the 5′ UTR of the transcript, we replaced the entire sequence upstream of the AUG of the uORF with the promoter, upstream regulatory region and 5′UTR from a *var* gene on chromosome 12 (PF3D7_1200100). Parasites transfected with this construct (V12uB, Figure 4I) displayed no repression. In addition, a 10 bp change to the sequence immediately upstream of the start codon of the uORF similarly disrupted repression (V2cB, Figure 3A). Thus in all cases, repression of translation of the dORF appears to be exquisitely sensitive to the sequence of all three upstream elements, including the 5′UTR, the uORF and the ICR.

Trans features of *var2csa* reinitiation mechanism

To detect the possible existence of trans acting factors that might contribute to the reinitiation mechanism, we co-transfected parasites with two plasmids. The first was the original V2B (Figure 2B), in which blasticidin pressure results in selection of parasites that are actively translating the dORF. The second plasmid also includes the *var2csa* upstream region, but contains a luciferase reporter gene in the dORF position (Figure 5A). In the co-transfected lines, it is thus possible to select for efficient translation of the dORF from transcripts from V2B, then determine how this selection affects translation of the dORF (encoding luciferase) on transcripts from the second construct. If a transacting factor required for reinitiation is limiting, the two transcripts will compete for this factor, reducing the efficiency of reinitiation for both dORFs. Such competition should be reflected in a greater amount of time required to select blasticidin resistant parasites and lower levels of luciferase expression. If however blasticidin pressure selects for parasites that are more efficient in translation reinitation in general, luciferase expression should be enhanced in parasites that have been selected by blasticidin. Using slightly different plasmid constructs, we observed evidence that both of these phenomena contribute to *var2csa* translational regulation.

A limiting factor is required for translation reinitiation at the dORF—Parasites were co-transfected with V2B and V2uGU (Figure 5A). V2uGU is identical to V2B except that the *bsd* selectable marker was replaced with the *Gaussia luciferase* coding region, and the marker gene used to select initial transfectants was changed from *dhfr* to *dhodh*. When co-transfected parasites were placed under blasticidin pressure, it took 22 additional days for the culture to reach 5% parasitemia (Figure 5A), compared to only 8 days when V2B was singularly transfected (Figure 2B). Similarly, luciferase expression was reduced when the co-transfected parasites were selected for blasticidin resistance (Figure 5A). These observations suggest that transcripts encoding the two dORFs (*bsd* and luciferase) compete for a limiting factor that enables them to efficiently reinitiate translation at the AUG of the dORF.

To further investigate trans effects on translation of the dORF, we co-transfected parasites with V2B and V2mGU (Figure 5B). V2mGU is identical to V2uGU except that a single bp mutation has been inserted within the start codon of the uORF that abolishes it as a start site for translation. These parasites expressed high levels of luciferase since translation of the dORF (encoding *Gaussia luciferase*) does not require reinitiation. Selection of the co-transfected parasites with blasticidin required only 8 additional days to reach 5% parasitemia, the same amount of time required by parasites transfected only with V2B, indicating that these transcripts do not compete for the ability to translate *bsd*. If translation of the uORF is disrupted (as it is in V2mGU), the transcripts no longer compete for the factor. Further, luciferase expression actually increased in the co-transfected lines upon selection with blasticidin, suggesting that translation initiation at the dORF position (either *bsd* or luciferase) becomes more efficient. It is possible that translation initiation in general

becomes more efficient when *bsd* expression is selected for, resulting in correspondingly higher levels of luciferase expression.

Measurements of *bsd* transcript levels in the co-transfected parasites shed some light on the nature of the competition between transcripts. When parasites were co-transfected with V2B and V2uGU, selection for blasticidin resistance resulted in a dramatic increase in *bsd* transcript levels (Figure 5A), suggesting the parasites require significantly more V2B transcript when the transcripts are competing for the ability to reinitiate translation at the *bsd* encoding dORF. Selection of parasites co-transfectected with V2mGU did not result in nearly as dramatic an increase in *bsd* transcripts (Figure 5B), providing additional data indicating that the V2mGU transcript does not compete with V2B transcript for a limiting factor required to express the dORF. Thus it appears that the limiting factor is only required for reinitiation of translation after the ribosome has completed translating an uORF.

DISCUSSION

The study of malaria in pregnancy has provided researchers with perhaps the best example for how PfEMP1 expression, cytoadhesion and immunity interact to determine the severity of disease (Salanti *et al.*, 2004). VAR2CSA is also the most advanced model for a PfEMP1 based, syndrome specific vaccine (Fried *et al.*, 2013). In addition, the unusual bicistronic structure of the *var2csa* transcript provides a unique opportunity to study how malaria parasites control translation. The work described here provides the first systematic and detailed analysis of mRNA sequence and structural aspects that influence the efficiency of translation initiation, as well the relatively unusual condition where translation initiates a second time on a single transcript. It is not yet clear if regulatory uORFs and/or bicistronic mRNAs are common in Plasmodium, however it is possible that the characteristics defined for *var2csa* translation could provide a model for other genes whose expression is regulated at the level of translation.

A simple switch from translational repression to efficient reinitiation

Given the data presented here, a model emerges for a simple translational switch that controls VAR2CSA expression. The presence of the uORF upstream of the VAR2CSA coding region ensures that the ribosome will complete an entire round of protein synthesis, including both initiation and termination of translation, prior to reaching the *var2csa* start codon. Thus, only if the ribosome retains initiation factors will it be able to reinitiate a second round of translation and expression of VAR2CSA. For the mechanism to work as a sensitive switch, reinitation should be very inefficient in the default state, resulting in strong repression of VAR2CSA expression. However, the system should be poised near the threshold for efficient reinitation, and thus able to switch quickly to translation of the VAR2CSA encoding dORF. The work described here provides insights into some of the molecular details of this switching mechanism.

A two component switching mechanism

Our data suggest that the mechanism underlying the switch between translational repression and efficient reinitiation consists of two separable components. The first component includes the uORF and the requirement for the ribosome to initiate translation at the start codon first encountered by the scanning ribosome. This structure ensures that a complete ribosomal complex, including both the large and small subunits as well as initiation factors, is engaged in active translation as it moves along the length of the transcript (see Figure 6A). Whether or not the ribosome will reinitiate translation at the dORF (encoding VAR2CSA) depends on whether initiation factors remain associated with the ribosome complex when it encounters the AUG of the dORF. Any conditions that reduce the retention of initiation

factors, for example increased length of the uORF or slowed transit time as the ribosome moves along the transcript, will correspondingly reduce the rate of translational reinitiation (Kozak, 2001). In the case of the *var2csa* transcript, the uORF appears to be very near the length where the ribosome tends to shed initiation factors. Any increase in the length of the uORF dramatically decreases the efficiency of reinitiation (compare V2B to V2GB and V2RB, Figures 2B, 3B and 3C, respectively), even in the presence of additional modifications that alleviate repression in the wildtype upstream region (for example compare V2cB to V2cGB, Figures 3A and 3D). However the uORF alone, when removed from the surrounding sequences, displays very little repression of reinitiation (see Figure 4E). Thus while translation of the uORF is required for translational repression of VAR2CSA expression, it alone is not sufficient.

The second component of the switching mechanism is dependent on the nucleotide sequence of the *var2csa* transcript. Sequence alterations to the ICR, uORF or 5′ UTR all disrupt repression (see Figures 4A-E, H and I, for example), suggesting that the native sequence reduces the propensity of the ribosome to reach the start codon of the dORF while retaining the initiation factors needed for reinitiating a second round of translation. Given that sequence specific RNA secondary structures can slow the movement of ribosomes along a transcript, and that increased transit time can reduce the retention of initiation factors by the ribosome complex (Kozak, 2001), an attractive model for the switching mechanism emerges. In the repressed state, the ribosome complex initiates translation at the start codon of the uORF, however elongation is slowed by sequence dependent aspects of the transcript, resulting in shedding of initiation factors from the ribosome complex prior to when it reaches the start codon of *var2csa* (Figure 6A). Thus VAR2CSA expression is repressed. In the event of a switch to the de-repressed state, initiation factors are either more stably associated with the ribosome complex (Figure 6B) or the movement of the ribosome through the uORF becomes more efficient (Figure 6C). Either or both conditions would enable the ribosome to reach the start codon of the *var2csa* region while still retaining initiation factors, thereby inducing expression.

The role of initiation factors

Translation reinitiation has been described in several systems (Kozak, 2002; Jackson, Hellen, and Pestova, 2012), in particular viruses (Powell, 2010), and these examples provide possible parallels to translation of the two ORFs within the *var2csa* transcript. In general, retention of initiation factors provides the key to efficient translation of the protein encoded by the dORF. For example, the virus *Helminthosporium victoriae* can reinitiate translation after translating a very long uORF (2315 bp), a property that is induced by a sequence motif found near the 3′ end of the uORF (Li *et al.*, 2011). This motif binds to the initiation factor eIF3, thereby directly increasing reinitiation frequency (Poyry *et al.*, 2007). Retention of eIF3 can also be mediated by the viral protein transactivator viroplasmin (TAV) in plant pararetroviruses (Futterer and Hohn, 1991). TAV tethers eIF3 to the elongating ribosome during uORF translation, thus ensuring that it is stably associated with the ribosome when it reaches the start codon of the dORF (Schepetilnikov *et al.*, 2011). These two examples show how both transcript sequence and transacting proteins can influence the rate of reinitiation, nonetheless each functions by influencing the retention of initiation factors. In the case of VAR2CSA expression, we similarly observed evidence for both transcript sequence and transacting factors that influence reinitiation frequency. We were unable to identify any proteins encoded in the *P. falciparum* genome with similarity to TAV, however the evidence for a limited factor observed in the co-transfection studies suggests that a protein with similar function might exist. Alternatively, the limiting factor could be a modified version of an initiation factor itself, for example by phosphorylation, that could influence its propensity to be retained by the ribosome and promote reinitiation.

The role of the *var2csa* sequence

Despite the simplicity of a linear ribosomal scanning/reinitiation model as an explanation for uORF-mediated control of dORF expression, the translational regulation of *var2csa* might be more complex. In our experiments, reinitiation of translation at the dORF was very efficient for the majority of bicistronic constructs tested. The exceptions were: plasmids containing longer uORFs than the *var2csa*-uORF, which can be explained by the probable loss of initiation factors during elongation; and the original construct with the entire, unmodified 5′ *var2csa*-sequence (V2B). It is possible that the 5′ *var2csa*-sequence influences reinitiation efficiency through either three-dimensional stem loop structures or by the presence of binding sites for regulatory *trans*-acting factors. We were not able to identify short, specific sequences within the upstream regions as would be predicted for protein binding sites. Rather, our results showed that 5′UTR, uORF and ICR sequences are all important to maintain repression of dORF translation, perhaps by acting together.

Experiments in which the elements were used separately did not display any repression of reinitiation. Therefore, a protein-binding site depending only on a specific sequence motif appears unlikely. Instead, the data suggest that a secondary structure is formed involving a large portion of the 5′ *var2csa*-sequence or specific parts of the three regions of the mRNA 5′ leader. The sequences of all three regions are highly conserved between different *P. falciparum* isolates and also with *P. reichenowi*, suggesting a function for these elements that requires maintenance of the primary nucleotide sequence. A three-dimensional loop structure would slow the traversal by ribosomes, thereby increasing the transit time of the ribosome and reducing reinitiation frequency (Kozak, 2002). The role of secondary structure based on sequence elements is now well established as a mechanism that influences ribosome transit efficiency and the likelihood of translational reinitiation (Kozak, 2005). This model predicts that since a specific structure is required to slow the ribosome, any alterations to the primary sequence of the upstream leader, including sequence duplications or changes in spacing of sequence elements, would disrupt the repressive secondary structure and increase the frequency of reinitiation. This model provides a plausible explanation for the unexpected observation that nearly all of the sequence alterations made to the leader sequences of our constructs, with the exception of those the lengthen the uORF, disrupted translational repression.

uORFs as regulators of protein expression

uORFs are common and tend to be conserved among species (Neafsey and Galagan, 2007). 35-49% of human and rodent transcripts have uORFs identified bioinformatically (Matsui *et al.*, 2007). The frequency of uORFs has not been rigorously analyzed in *P. falciparum*, but a survey of the *P. falciparum* genome database found that 265 genes (4.8% of *P. falciparum* genes) have a uORF with similar features to the *var2csa*-uORF (minimum uORF length: 200 bp; minimum ICR length: 50 bp; maximum uORF+ICR length: 800 bp) (J. Tan, personal communication). Thus regulating translation by controlling reinitiation frequency could be relatively common. uORFs are thought to function in cellular genes to control expression of downstream protein-encoding ORFs in response to cellular environment (Morris and Geballe, 2000), or as a way of limiting the synthesis of potent proteins that would be harmful if overproduced (Kozak, 2002). It is tempting to propose that conditions or factors specific to the placental environment could induce VAR2CSA expression. For example, the placenta is known to be rich in polyamines, molecules that can influence the efficiency of translation. However addition of polyamines or hormones known to be abundant during pregnancy have thus far failed to induce VAR2CSA expression in our parasite cultures (Amulic *et al.*, 2009).

In summary, we suggest a model for translational regulation of VAR2CSA expression whereby in non-pregnant conditions, ribosomes translating through the uORF encounter a highly structured region comprising the uORF, ICR and sequences within the 5′UTR. Transit through this region is slowed, allowing most initiation factors to disassociate from the ribosomes and thereby strongly repressing reinitiation. Therefore, the majority of parasites do not express VAR2CSA and do not activate the immune memory of the host. In the presence of a placenta, a small number of parasites are able to retain initiation factors with the ribosomes and efficiently express VAR2CSA, thus enabling them to take advantage of the placental environment. The selective advantage provided to these parasites promotes their proliferation within the parasite population. The key to the switch in VAR2CSA expression lies in the ribosomes ability to translate the uORF, keep initiation factors tethered to the ribosome complex, and to reinitiate translation at the start codon of the dORF. This likely involves a limited, *trans*-activator factor that tethers initiation factors to the ribosome or a molecule that unfolds the highly structured upstream region of the transcript. Future work will be directed toward testing this model and further characterizing the details of the translational switching mechanism.

Experimental Procedures

Plasmid constructs

All plasmids used in this study are derivatives of the previously described V2B, V2mB and V2RB (Amulic *et al.*, 2009). The *Gaussia luciferase* sequence was amplified from pCMV-GLuc (New England BioLabs) and was cloned into the NcoI-ClaI sites of V2RB. For the V2dsB construct, a different uORF-sequence encoding the same amino acids with alternative codons and a “scrambled” ICR-sequence with the same AT content as the *var2csa*-ICR were synthesized by Genewiz and cloned into NcoI-HpaI sites of V2B. The varChr12 promoter was obtained from the 3D7 genome by amplifying 2 kb upstream of the *var* gene PF3D7_1200100 and cloning the fragment between the AatII-NcoI sites of V2B plasmid. The yeast *dihydroorotate dehydrogenase* (y*dhodh*) gene was amplified from pUF-1 (Ganesan *et al*., 2011) and cloned into the NotI-SacI sites of V2B. Other constructs were made from combinations of these fragments and plasmids. Site-directed mutagenesis was performed using the Quickchange kit (Stratagene). Supplemental Table 2 describes the detailed cloning procedures used to obtain every construct.

Parasite culture and transfection

Plasmodium falciparum parasites (the 3D7 line) were obtained from MR4 (MRA-156, MR4, ATCC). Parasites were cultured according to standard procedures in media containing Albumax II (Gibco) without human serum. Parasites were incubated at 37°C in an atmosphere of 5% oxygen, 5% carbon dioxide, and 90% nitrogen. Constructs were transfected into parasite using “DNA-loaded” RBCs as previously described (Deitsch, Driskill, and Wellems, 2001). Stable transfected parasites were cultured in media containing 4 nM WR99210 (Jacobus Pharmaceuticals) for hDHFR-selectable marker (Fidock and Wellems, 1997) or 1.5 μM DSM1 (Vaidya A.B. laboratory, Drexel University, Pennsylvania, USA) for parasites harboring the yDHODH selectable marker (Ganesan *et al.*, 2011).

Growth curves assay

Transfected parasites lines were synchronized to ring stages using either percoll-sorbitol gradient centrifugation (Aley, Sherwood, and Howard, 1984) or alanine lysis (Braun-Breton, Rosenberry, and Da Silva, 1988). 10 ml of synchronized-parasites at 0.075% parasitemia and 5% hematocrit were divided in two 25 cm^2-culture flasks. One flask was cultured with Blasticidin S HCl (Gibco) (5 μg/ml) and the other without. Parasites were cultured with

complete media supplemented with the selectable drug (WR99210 or DSM1) on an orbital shaker. Parasitemia was determined by Giemsa-stained smears by counting 1000-2000 erythrocytes per culture. Assays were finished when 5% parasitemia was reached. A growth assay with the V2B transgenic parasite line was always performed in parallel with the transgenic line assessed. We performed at least two independent experiments for each parasite line.

DNA extraction, plasmid rescue and Southern blot analysis

Genomic DNA was extracted from parasites by a standard phenol/chloroform and ethanol-precipitation method (Swamy, Amulic, and Deitsch, 2011). To ensure experiments were done with the correct episomal plasmids, plasmid rescue and sequencing were performed after growth curves assays for all the transfected lines. For plasmid rescue, 500 ng of parasite genomic DNA were used to transform XL-10 Gold (Stratagene) *E. coli*-competent cells. Southern blot analysis was used to verify that transfected plasmids remained episomal and was performed as described previously (Swamy, Amulic, and Deitsch, 2011).

RNA extraction and quantitative PCR analysis

RNA was extracted from synchronized ring stage parasites 24 h after isolation of schizonts using percoll-sorbitol gradient centrifugation (Aley, Sherwood, and Howard, 1984). RNA extraction was performed using Trizol LD Reagent (Invitrogen) as described (Kyes, Pinches, and Newbold, 2000). RNA was purified using the PureLink RNA Mini Kit (Ambion) according to manufacturer's protocol and afterwards treated with DNase I (Invitrogen). cDNA synthesis was performed with Superscript II RNase H reverse transcriptase (Invitrogen) with random primers (Invitrogen) as described by the manufacturer. 800 ng of total RNA were used for each cDNA synthesis reaction and a control reaction without reverse transcriptase was performed in parallel.

Quantitative PCR (qPCR) analysis was done using the relative standard curve method. qPCR was performed using cDNA as template. All qPCR reactions were performed in triplicate with an ABI Prism 7900HT (Applied Biosystems) using iTaq SYBR Green Supermix (Bio-Rad) and the primers listed in Supplemental Table 1. Expression was normalized to amounts of the control gene *seryl tRNA synthetase*.

Luciferase activity assay

Schizont-stage parasites were isolated using percoll-sorbitol gradient centrifugation (Aley, Sherwood, and Howard, 1984) and were used to inoculate a culture. Luciferase activity was measured 24 h after cultures were initiated. iRBCs were obtained from 200 μl of culture by centrifugation, and the cells were lysed by the addition of *Renilla* Lysis Buffer (Promega). Supernatants were assayed for *Gaussia* and *Renilla* activities using the Luciferase Assay System from Promega and a TD-20/20 luminometer (Turner Designs). Luciferase activity is expressed as luminescence units per 1% parasitemia and it is normalized per mRNA levels. The luciferase activity of each clonal line was determined in three independent experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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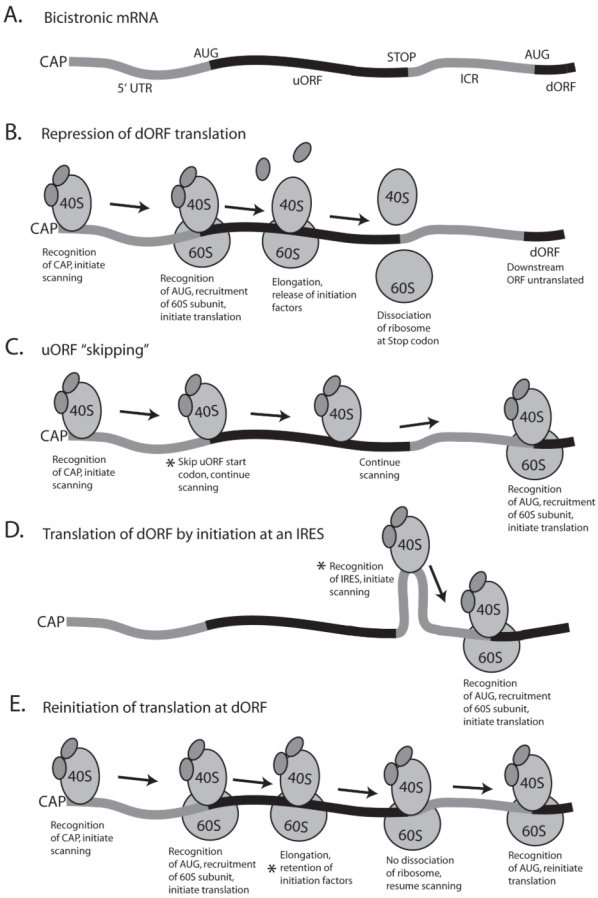

Figure 1. Typical structure of a bicistronic transcript and mechanisms that allow downstream ORF translation

(A) The basic structure of a bicistronic mRNA containing a CAP, 5′ untranslated region (5′UTR), an upstream ORF (uORF), an intercistronic region (ICR) and a downstream ORF (dORF) is shown. The start codons (AUG) and stop codon (STOP) are also identified. *(B)* The ribosome will typically recognize and translate the uORF. Once it reaches the stop codon, it will dissociate, thus preventing the dORF from being translated into protein. The usual repression of initiation at the start codon of the dORF can be circumvented by different mechanisms: *(C)* uORF "skipping" and recognition of a downstream start codon in a more optimal context, *(D)* translation of dORF by initiation at an internal ribosomal entry site (IRES) and *(E)* reinitiation of translation at dORF after first translating the uORF.

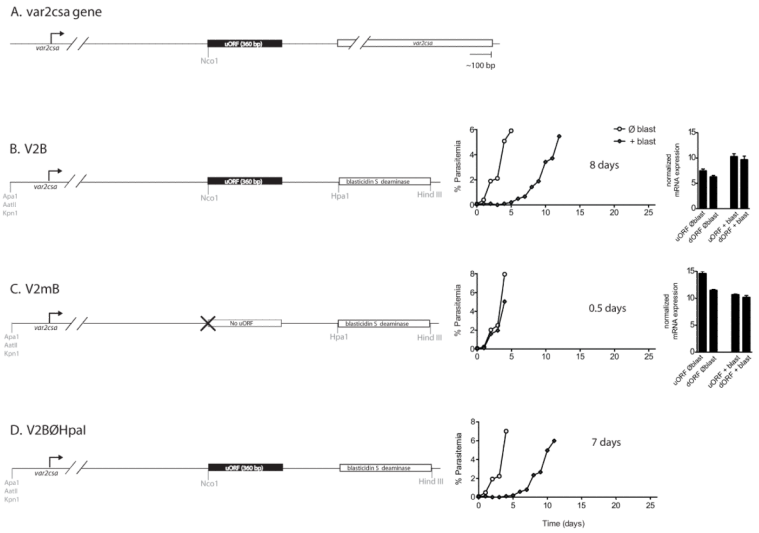

Figure 2. Selection of parasites translating the dORF of the *var2csa* bicistronic transcript

(A) The structure of the *var2csa* gene is shown. The transcription start site is marked with an arrow, the upstream ORF (uORF) is shown as a solid box, and the VAR2CSA coding region is shown as an empty box. *(B)* The construct V2B includes the transcription start site and regulatory region, the intact uORF and the intercistronic region from the *var2csa* gene, but the VAR2CSA coding region has been replaced by the coding region of the *blasticidin S deaminase* gene (*bsd*). The middle panel shows growth curves of parasites cultured with and without blasticidin (Ø/+ blast). The additional time (in days) required for the parasites to reach 5% parasitemia under blasticidin selection compared to the parasites cultured without the drug is shown. The right panel shows quantitative RT-PCR measurements of steady state mRNA levels for both the uORF and *bsd* (dORF) regions of the transcript from parasite grown with or without blasticidin (5 μg/ml). *(C)* V2mB is identical to V2B, except a single base pair mutation in the uORF start codon has abolished it as a start site for translation. Growth curves and quantitative RT-PCR measurements are also shown. *(D)* V2BØHpaI is identical to V2B except the HpaI site near the initiation codon of the *bsd* open reading frame has been removed by site-directed mutagenesis to restore the wildtype sequence found at this position in the *var2csa* gene. Growth curves with and without blasticidin are very similar to those observed for V2B transfected parasites. Restriction sites used for construction of the plasmids are shown.

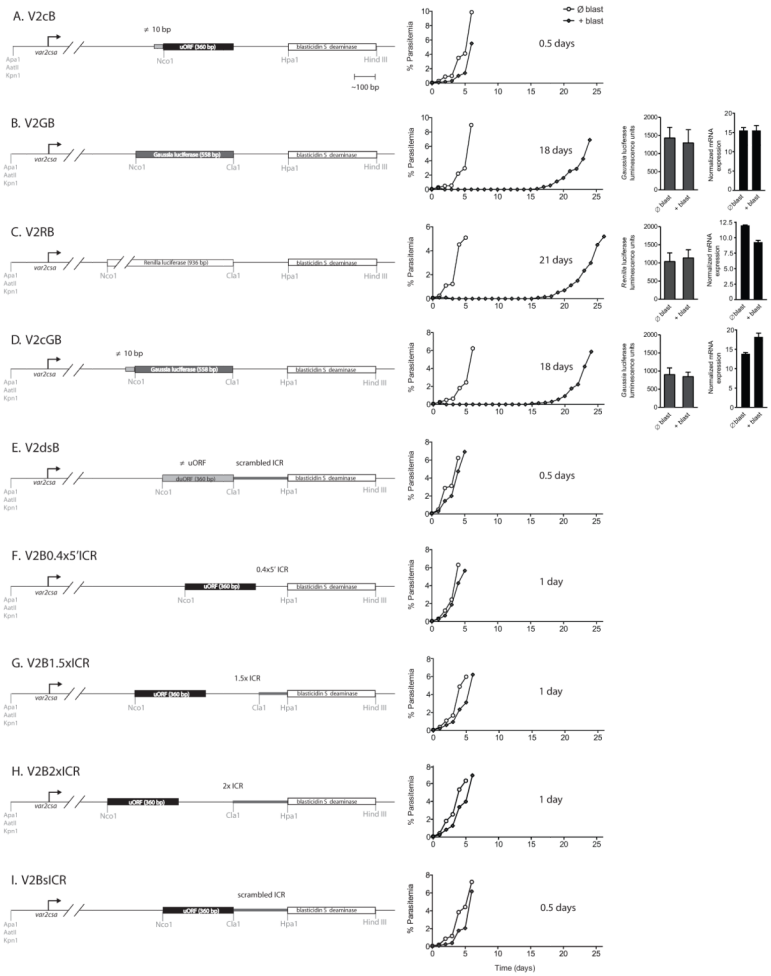

Figure 3. uORF and ICR requirements for dORF translational repression

Parasites were stably transfected with plasmid constructs and selected for their ability to translate the dORF encoding *blasticidin S deaminase* (*bsd*). Schematic diagrams of the constructs are shown on the left along with corresponding growth curves of parasites cultured with and without blasticidin (5 μg/ml). The additional time (in days) required for the parasites to reach 5% parasitemia under blasticidin selection compared to parasites cultured without the drug is shown. V2cB *(A)* was made by replacing the 10 bp upstream of the start codon of the uORF with the analogous sequence found immediately upstream of the dORF start codon. In V2GB and V2RB *(B, C)* the uORF element was replaced with the coding regions of the reporter genes *Gaussia* and *Renilla luciferase*. V2cGB *(D)* has the 10 bp upstream of the start codon of the uORF replaced by the sequence found immediately upstream of the dORF start codon and the *Gaussia luciferase* coding region in the place of uORF. For V2GB, V2RB and V2cGB *(B-D)*, luciferase expression levels and steady state RNA levels are also shown and demonstrate that translation of the uORF remains steady after selection of blasticidin resistant parasites. Parasites were cultured with or without blasticidin pressure and synchronized to ring stages. Luciferase activity was normalized to levels of mRNA expression. In V2dsB *(E)* the uORF sequence was replaced with a divergent sequence that encodes the same amino acids, but that uses alternative codons, and the ICR sequence was “scrambled”. V2B0.4×5′ICR, V2B1.5×ICR and V2B2×ICR (*F*, *G*, and *H*, respectively) are constructs with altered ICR length to 0.4, 1.5 and 2 times the length of the region found in the endogenous *var2csa* transcript. V2B0.4×5′ICR *(F)* contains the 5′

0.4 portion of the ICR. In V2B1.5×ICR and V2B2×ICR *(G, H)* the ICR length was increased adding the scrambled sequence used in V2dsB *(E)*. In V2BsICR *(I)* the ICR was replaced by a sequence of identical size and AT content, but with a scrambled sequence. Restriction sites used for construction of the plasmids are shown.

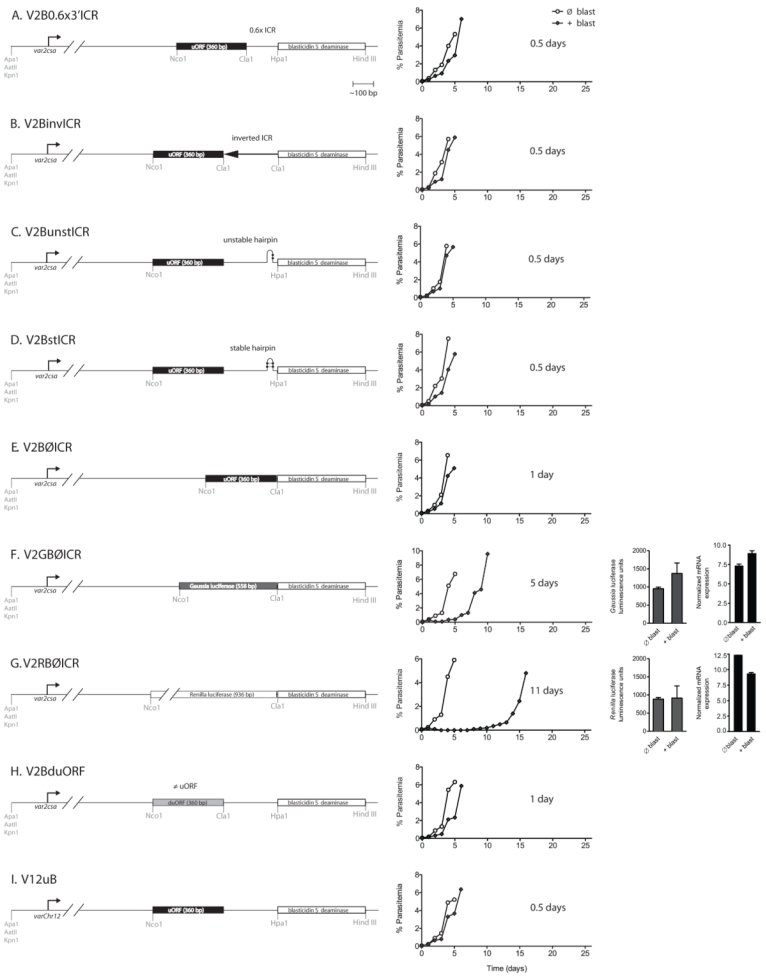

Figure 4. Sequence requirements for repression of dORF translation

Parasites were stably transfected with plasmid constructs and selected for their ability to translate the dORF encoding *blasticidin S deaminase* (*bsd*). Schematic diagrams of the constructs are shown on the left and corresponding growth curves of parasites cultured with and without blasticidin (5 μg/ml) are shown on the right. Assays were performed as described for Figure 3. The additional time (in days) required for the parasites to reach 5% parasitemia under blasticidin selection compared to parasites cultured without the drug is shown. V2B0.6×3′ICR *(A)* contains only the 3′ 0.6 of the ICR. In V2BinvICR *(B)* the ICR was inverted relative to its orientation in the *var2csa* transcript. V2BunstICR *(C)* is identical to V2B (Figure 2B), except 9 point mutations were introduced at critical sites of a predicted ICR hairpin with the goal of disrupting it. V2BstICR *(D)* is similar to V2BunstICR *(C)* but compensatory mutations were introduced that were predicted to re-establish the putative hairpin. In V2BØICR *(E)* the ICR element was completely removed. In V2GBØICR and V2RBØICR *(F* and *G)* the uORF was replaced with the coding regions of the reporter genes *Gaussia* and *Renilla luciferases* and the ICR element was removed. For these constructs, luciferase expression levels and steady state RNA levels are also shown (right). In each of these constructs (*E-G*) the two ORFs remain separate and the ORF in the upstream position contains an in frame stop codon. In V2BduORF *(H)* the uORF sequence was replaced with a divergent sequence that encoded the same amino acids, but that used alternative codons. V12uB *(I)* was made by replacing the entire sequence upstream of the AUG of the uORF

with the promoter, upstream regulatory region and 5′UTR from a *var* gene on chromosome 12 (PF3D7_1200100). Restriction sites used for construction of the plasmids are shown.

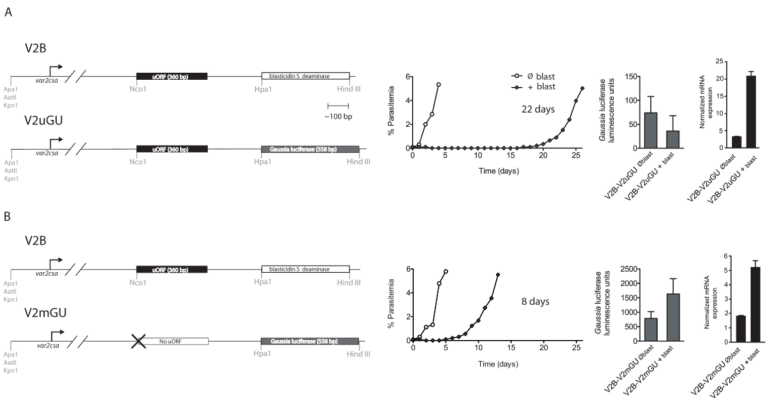

Figure 5. Trans acting factors are implicated in the *var2csa* reinitiation mechanism

Parasites stably co-transfected with two plasmids were used to study trans effects on translation of the dORF. Schematic diagrams of the plasmid constructs are shown on the left, growth curves with and without blasticidin pressure are shown in the center, *Gaussia* luciferase expression is shown in the central bar graph, and *bsd* mRNA levels are shown at the right. Selection of blasticidin resistant parasites co-transfected with V2B and V2uGU is delayed when compared to parasites transfected with V2B alone (see Figure 2B), and luciferase expression is reduced *(A)*. In contrast, parasites co-transfected with V2B and V2mGU behave similarly to parasites transfected only with V2B *(B)*. In addition, luciferase expression increased in parasite populations after blasticidin resistance was established. Selection with blasticidin results in a dramatic increase in transcript levels of V2B in parasites co-transfected with V2uGU, while a much small increase is detected in parasites co-transfected with V2mGU. This suggests that the V2B transcript competes with the V2uGU transcript for the ability to translate the dORF and consequently significantly more transcript is required, while it does not compete (or competes much less) with the V2mGU transcript. Restriction sites used for construction of the plasmids are shown.

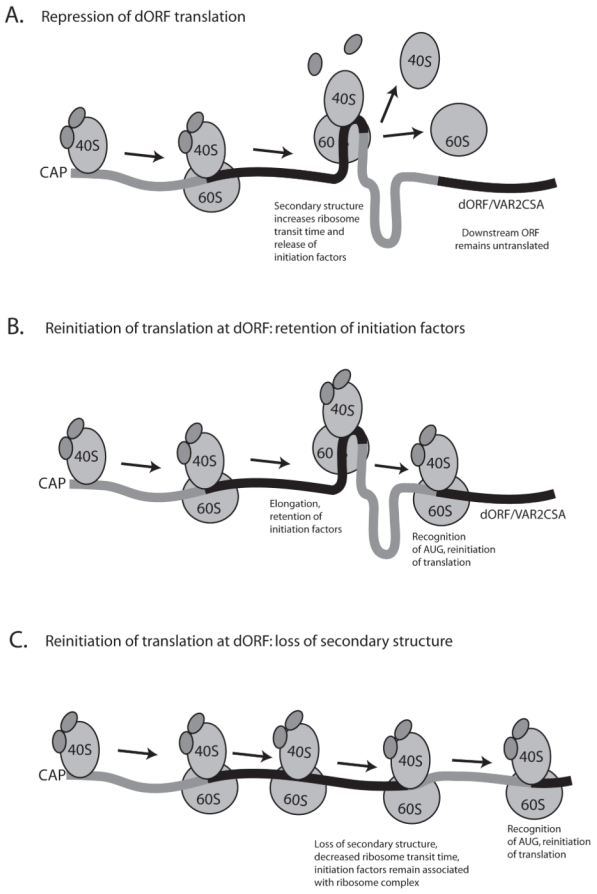

Figure 6. A model for control of VAR2CSA translation

Under standard conditions *(A)*, the 40S subunit and initiation factors begins scanning at the CAP and initiates translation when it reaches the start codon of the uORF. Sequence dependent secondary structure slows transit time of the translating ribosome, allowing nearly complete release of initiation factors from the ribosome complex. In the absence of initiation factors, the ribosome dissociates from the transcript upon reaching the stop codon of the uORF, thus preventing translation of the dORF encoding VAR2CSA. If initiation factors are more stably associated with the ribosome while it is translating the uORF *(B)*, the ribosome can resume scanning after it passes the uORF stop codon and reinitiate a second round of translation when it encounters the start codon of the dORF. If the secondary structure of the transcript is disrupted *(C)*, transit time of the translating ribosome through the uORF is reduced and initiation factors are less likely to be shed. This enables the ribosome to resume scanning when it reaches the ICR and increases the efficiency of a second round of translation.