International Journal for Parasitology 39 (2009) 1425–1439

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

A *var* gene promoter implicated in severe malaria nucleates silencing and is regulated by 3′ untranslated region and intronic *cis*-elements

Rebecca A. Muhle [a], Sophie Adjalley [b], Brie Falkard [b], Louis J. Nkrumah [a], Michael E. Muhle [c], David A. Fidock [b,d,*]

[a] Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, NY 10461, USA
[b] Department of Microbiology, Columbia University College of Physicians and Surgeons, Room 1502, Hammer HSC. 701 W 168th Street, New York, NY 10032, USA
[c] Univation LLC, Houston, TX 77056, USA
[d] Department of Medicine, Columbia University College of Physicians and Surgeons, New York, NY 10032, USA

ARTICLE INFO

Article history:
Received 10 October 2008
Received in revised form 27 April 2009
Accepted 5 May 2009

Keywords:
Plasmodium falciparum
var
Antigenic variation
Gene regulation
Multivariate analysis

ABSTRACT

Questions surround the mechanism of mutually exclusive expression by which *Plasmodium falciparum* mediates activation and silencing of *var* genes. These encode PfEMP1 proteins, which function as cytoadherent and immunomodulatory molecules at the surface of parasitised erythrocytes. Current evidence suggests that promoter silencing by *var* introns might play a key role in *var* gene regulation. To evaluate the impact of *cis*-acting regulatory regions on *var* silencing, we generated *P. falciparum* lines in which *luciferase* was placed under the control of an UpsA *var* promoter. By utilising the Bxb1 integrase system, these reporter cassettes were targeted to a genomic region that was not in apposition to *var* subtelomeric domains. This eliminated possible effects from surrounding telomeric elements and removed the variability inherent in episomal systems. Studies with highly synchronised parasites revealed that the UpsA element possessed minimal activity in comparison with a heterologous (*hrp3*) promoter. This may result from the integrated UpsA promoter being largely silenced by the neighbouring *cg6* promoter. Our analyses also revealed that the DownsA 3′ untranslated region further decreased the luciferase activity from both cassettes, whereas the *var* A intron repressed the UpsA promoter specifically. By applying multivariate analysis over the entire cell cycle, we confirmed the significance of these *cis*-elements and found the parasite stage to be the major factor regulating UpsA-promoter activity. Additionally, we observed that the UpsA promoter was capable of nucleating reversible silencing that spread to a downstream promoter. We believe these studies are the first to analyse promoter activity of Group A *var* genes, which have been implicated in severe malaria, and support the model that *var* introns can further suppress *var* expression. These data also suggest an important suppressive role for the DownsA terminator. Our findings imply the existence of multiple levels of *var* gene regulation in addition to intrinsic promoter-dependent silencing.

1. Introduction

The *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP1) variant surface antigens encoded by the *var* gene family (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995) have repeatedly been linked to the immuno-evasive strategies and cytoadherent phenotypes of parasitised erythrocytes (Biggs et al., 1992; Roberts et al., 1992; Bull and Marsh, 2002). The etiological agent of severe malaria, the *P. falciparum* parasite, activates the expression of only one PfEMP1 ligand at a time, thereby mediating binding to a variety of host endothelial cell surface receptors, including Cluster of Differentiation 36 (CD36) or Inter-Cellular Adhesion Molecule 1 (ICAM-1) in the host microvasculature, and chondroitin sulphate A (CSA) in the placenta (Fried and Duffy, 1996; Andrews et al., 2003; Salanti et al., 2003). This process sequesters the parasite, preventing immune-mediated clearance in the spleen (Barnwell, 1989).

Each *var* gene is defined by the possession of one or more combinations of multiple adhesive domains in a two-exon gene structure, with ~60 different *var* genes per haploid genome (Smith et al., 2000; Gardner et al., 2002). The entirety of the global *var* gene repertoire is vast, however, as different parasite isolates may possess a wholly unique array of *var* genes (Trimnell et al., 2006; Barry et al., 2007; Kraemer et al., 2007). Sequencing of clonal laboratory isolates revealed that *var* genes can be classified into several subfamilies, primarily Groups A, B and C. This is based on their chromosomal location, the nucleotide identity of their non-

* Corresponding author. Address: Department of Microbiology, Columbia University College of Physicians and Surgeons, Room 1502, Hammer HSC. 701 W 168th Street, New York, NY 10032, USA. Tel.: +1 212 305 0816; fax: +1 212 305 4038.
E-mail address: df2260@columbia.edu (D.A. Fidock).

0020-7519/$36.00
doi:10.1016/j.ijpara.2009.05.001

coding regions and the adhesive domains encoded within the protein – in particular the "head structure" made up of the paired amino-terminal Duffy-binding-like α (DBLα) and cysteine-rich interdomain region α (CIDRα) domains (Gardner et al., 2002; Kraemer and Smith, 2003; Lavstsen et al., 2003). Group B and C genes comprise the bulk of the *var* family in each genome, with Group B genes located subtelomerically and transcribed away from the telomere and Group C genes found in several tandem arrays in central chromosomal locations. Group A genes are also present in subtelomeric regions but in a tail-to-tail orientation with the Group B genes such that they are transcribed towards the telomere.

var groups also possess highly specific upstream non-coding regions, such that UpsA, UpsB and UpsC promoters precede Group A, B and C *var* coding sequences, respectively. The presence of a few transitional *var* genes represented by Group B/A (UpsB with a Group A head structure) and Group B/C (Ups B in a central chromosomal location) strengthens the hypothesis that these groupings are maintained by intragroup recombination with occasional intergroup mixing (Kraemer et al., 2007; Bull et al., 2008). The relevance of these classifications to clinical infection is apparent in the stratification of the different *var* gene subfamilies to different pathologies, such that clinical isolates obtained from children with severe malaria express more Group A and B *var* genes (Kaestli et al., 2006; Kyriacou et al., 2006; Rottmann et al., 2006), while placental isolates express a single *var* gene, var*2csa* (Tuikue Ndam et al., 2005; Magistrado et al., 2008).

To date, the *var* intron has been the only *cis*-acting sequence element implicated in the modulation of *var* promoter function. Transient transfection studies have documented the repression of a *var* promoter-driven reporter gene by a closely apposed *var* intron (Deitsch et al., 2001). This intron-mediated silencing was found to be dependent on a bidirectional promoter activity that originated from the central region of the intron (Calderwood et al., 2003; Gannoun-Zaki et al., 2005) and required a one-to-one pairing of one *var* promoter with one intron (Frank et al., 2006). However, this model of intron-mediated *var* silencing has been controversial, as the presence or absence of the *var* intron was reported to have no effect on the silencing of a *var* promoter driving a selectable marker (Voss et al., 2006, 2007). In those studies, parasites were able to recognise an intron-less *var* promoter as part of the *var* gene family, suggesting that all information necessary for silencing and mutually exclusive expression might reside within the *var* promoter sequence. These differing accounts were addressed by a subsequent report, demonstrating that various heterologous promoters had the same silencing effect on an upstream *var* promoter as did the *var* intron (Dzikowski et al., 2007). This implied that silencing of *var* expression only required a promoter activity within the vicinity of the *var* promoter.

Exactly how a second paired promoter might allow the parasite to recognise and "count" a *var* promoter remains unclear, but there appears to be a requirement for a specific nuclear element that can be titrated away by a highly active "uncounted" *var* promoter (Dzikowski and Deitsch, 2008). The intron has also been shown to insulate a downstream selectable marker from the spread of *var* promoter activation, which suggests that the intron is able to affect the epigenetic status of chromatin surrounding the *var* promoter (Voss et al., 2006).

While alignments of *var* promoter sequences stratify these genes into distinct and consistent functional classes, the downstream non-coding regions of the Group A and B genes are also highly conserved, and are termed DownsA and DownsB (Kraemer and Smith, 2003; Lavstsen et al., 2003). The potential regulatory effects of these elements on *var* gene promoters have not been explored. Additionally, although Group A *var* genes represent a clinically significant subpopulation, the specific effects of the Group A intron on UpsA expression have not been assessed. Here, we report our investigations into Group A *var* gene regulation using the firefly *luciferase* reporter gene. Our data show that the UpsA promoter is minimally active in this integrated context. These studies have not definitively determined whether, in this genomic configuration UpsA is expressed at biologically relevant levels. Nevertheless, our multivariate analyses on tightly synchronised parasite lines provide evidence that the highly conserved Group A *var* 3′ untranslated region (UTR), referred to as DownsA, and the Group A intron, both decrease expression of a reporter produced from the UpsA *var* promoter, albeit with differing specificities. These findings, combined with previous studies of Group B and C *var* genes, suggest that the parasite possesses a consistent means of *var* family regulation, with additional control imposed by conserved subgroup-specific DNA *cis*-elements including the DownsA element.

2. Materials and methods

2.1. Parasite cultivation and transfection

Parasites were cultivated in human red blood cells at 3–4% haematocrit (Fidock et al., 1998). The previously described *P. falciparum* Dd2^{attB} parasite line, which contains an *attB* site within the disrupted locus of the *cg6* gene (Nkrumah et al., 2006), was grown continuously in the presence of 2.5 nM of the *Plasmodium* dihydrofolate reductase (*dhfr*)-specific antifolate inhibitor WR99210 (Jacobus Pharmaceuticals, Princeton, NJ). Predominantly ring-stage parasites at 4–8% parasitemia were electroporated with 50 μg of an *attP*-containing plasmid and 50 μg of the pInt plasmid, as previously described (Nkrumah et al., 2006). G418 sulphate (Cellgro) (100 μg/mL) and 2.5 μg/mL blasticidin S hydrochloride (BSD; Invitrogen) were added on day 2 post-transfection to select for co-transfected parasites. These typically became microscopically visible 17–26 days post-transfection. At that time, G418 was removed to eliminate the pInt plasmid from the culture. Cloning was performed by limiting dilution at 0.7 parasites per well (Goodyer et al., 1997).

2.2. Plasmid preparation

To generate the control p∂LH plasmid that contains the luciferase gene and the *hrp2* 3′ UTR but has no promoter driving luciferase expression, we removed the *hrp3* promoter of the luciferase expression plasmid pHLH (Wu et al., 1995) by *Asp*718 and NsiI digestion, and replaced this with an oligonucleotide linker prepared by annealing two primers: 620 (5′-GTACCAAAGTCGA CAAATGCA) and 621 (5′-TTTGTCGACTTTG). The pALH plasmid was generated by inserting a 2.2 kb UpsA fragment that was PCR amplified from the *var* gene PFD1235w using primers 772 (5′-AA AggtaccGTATGTTATACC) and 773 (5′-GCAgaatgcattcTTATAACAAA GTATTTAAATAA). These primers introduced 5′ KpnI and 3′ XmnI sites (lowercase) that were used to subclone into the KpnI and BfrBI digested p∂LH plasmid. A 0.75 kb fragment of the PFD1235w intron, amplified with primers 636 (5′-TTTctgcagGTAAATGGAGTAT ATATATGTG) and 774 (5′-CGGctgcagCTATAATTAAAAAAAAAGGTAT GTATG) that both contained a PstI site, was inserted into the PstI site of pALH and pHLH. This yielded the plasmids pALHi and pHLHi, respectively. The 1.4 kb DownsA region of PFD1235w was PCR amplified using primers 626 (5′-CGGATGTATGGAATATATaagcttAA AA) and 627 (5′-GATATctgcagTACTATTACATAATACATTC), and cloned into HindIII–PstI-digested pALH and pHLH, yielding pALA and pHLA. All PFD1235w sequences were amplified from genomic DNA of the *P. falciparum* 3D7 strain. Firefly luciferase expression cassettes were isolated from the vectors pALHi, pALA, pHLH,