EMBO reports

scientific report

Mechanisms underlying mutually exclusive expression of virulence genes by malaria parasites

Ron Dzikowski[1†], Felomena Li[1], Borko Amulic[1], Andrew Eisberg[1], Matthias Frank[2,3], Suchit Patel[1], Thomas E. Wellems[4] & Kirk W. Deitsch[1+]

[1]Department of Microbiology and Immunology, and [2]Department of International Medicine, Weill Medical College of Cornell University, New York, New York, USA, [3]Department of Parasitology, University of Tuebingen, Tuebingen, Germany, and [4]Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, USA

A fundamental yet poorly understood aspect of gene regulation in eukaryotic organisms is the mechanisms that control allelic exclusion and mutually exclusive gene expression. In the malaria parasite *Plasmodium falciparum*, this process regulates expression of the *var* gene family—a large, hypervariable repertoire of genes that are responsible for the ability of the parasite to evade the host immune system and for pathogenesis of the disease. A central problem in understanding this process concerns the mechanisms that limit expression to a single gene at a time. Here, we describe results that provide information on the mechanisms that control silencing and single gene expression and differentiate between several models that have recently been proposed. The results provide the first evidence, to our knowledge, supporting the existence of a postulated *var*-specific, subnuclear expression site and also reinforce the conclusion that *var* gene regulation is based on cooperative interactions between the two promoters of each *var* gene.

Keywords: malaria; antigenic variation; allelic exclusion; silencing; transcription

EMBO reports (2007) **8**, 959–965. doi:10.1038/sj.embor.7401063

INTRODUCTION

One of the most interesting yet poorly understood aspects of gene regulation in eukaryotic organisms is the process by which cells choose to express only a single member among many within a family of multiple, functionally equivalent genes. This type of gene regulation is responsible for allelic exclusion of immunoglobulin genes in B cells (Corcoran, 2005), single odorant receptor gene expression (Serizawa *et al*, 2004), genetic imprinting (Arney *et al*, 2001) and X-chromosome inactivation in female mammals (Okamoto *et al*, 2004). Although considerable progress has been made in recent years in understanding certain features of these regulatory pathways, many aspects remain ill-defined. In addition, most work has focused on mutually exclusive expression in higher eukaryotes, in particular, in model organisms such as mice or fruitflies.

Plasmodium falciparum, the parasite responsible for the most severe form of human malaria, invades circulating red blood cells, resulting in severe anaemia and circulatory disruption due to vascular obstruction. During its replication in the red blood cell, parasites modify the host cell, placing a cytoadherence protein called PfEMP1 on the infected cell surface (Kyes *et al*, 2001). Different antigenic forms of PfEMP1 are encoded by different members of the multicopy *var* gene family. PfEMP1 expression enables *P. falciparum*-parasitized red blood cells to sequester in the small venules of deep tissue vascular beds and avoid destruction by the spleen, while expression switches between individual *var* genes allow the parasites to evade antibody responses against PfEMP1 and thereby produce a persistent infection. Crucial to this process of cytoadhesion and antigenic variation is the absolute requirement that each individual parasite expresses only a single *var* gene at a time; for this, parasites have evolved a mechanism of strict, mutually exclusive, *var* gene expression analogous to that described for certain genes in higher eukaryotes. However, in contrast to expression patterns that are typically irreversible in the somatic lines of those systems, malaria parasites have the ability to repeatedly switch *var* gene expression during the course of an infection.

[1]Department of Microbiology and Immunology, and [2]Department of International Medicine, Weill Medical College of Cornell University, 1300 York Avenue, Box 62, New York, New York 10021, USA
[3]Department of Parasitology, University of Tuebingen, Wilhemstrasse 27, Tuebingen 72074, Germany
[4]Laboratory of Malaria and Vector Research, Twinbrook III Building, Room 3E-10D, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892-8132, USA
[†]Present address: Department of Parasitology, The Kuvin Center for the Study of Infectious and Tropical Diseases, The Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel
[+]Corresponding author. Tel: +1 212 746 4976; Fax: +1 212 746 4028;
E-mail: kwd2001@med.cornell.edu

Received 28 March 2007; revised 1 August 2007; accepted 1 August 2007; published online 31 August 2007

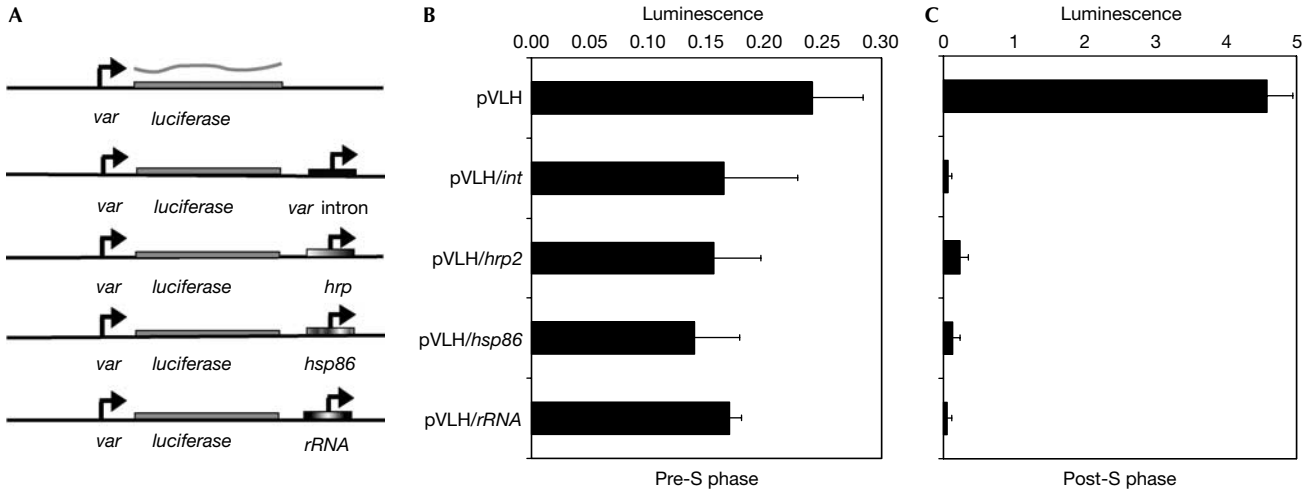

Fig 1 | An episomal *var* promoter is silenced by adjacent promoters. (**A**) Schematic maps of vectors. Plasmid constructs carry either a 'free' *var* promoter (pVLH) or a *var* promoter paired with a *var* intron promoter (pVLH/*int*), the *hrp2* promoter (pVLH/*hrp*), the *hsp86* promoter (pVLH/*hsp86*) or the *rRNA* promoter (pVLH/*rRNA*). Luciferase activity was measured in transfected synchronized parasites (**B**) before S phase, or (**C**) after proceeding through at least one S phase. S-phase dependence was determined as described previously (Calderwood *et al*, 2003). Error bars represent calculated standard deviations. *hrp2*, histidine-rich protein 2; *hsp86*, heat-shock protein 86; *rRNA*, ribosomal RNA.

Expression of *var* genes is regulated at the level of transcription initiation (Scherf *et al*, 1998), but little is understood of *var* promoter recognition or of the mechanisms by which mutually exclusive expression is controlled. One model to explain this process postulates that a *var*-specific, subnuclear expression site exists in the parasite nucleus, and that by limiting access to a single gene at a time, mutually exclusive expression is achieved (Merrick & Duraisingh, 2006). A similar hypothesis has been proposed for monoallelic variant surface glycoprotein (*vsg*) gene expression in African trypanosomes (Chaves *et al*, 1998; Navarro & Gull, 2001). Although this is an intuitively attractive model, until now it has not been possible to show whether such an expression site exists, or what role, if any, it might have in controlling *var* gene expression. Other proposed models have incorporated promoter–promoter interactions or the involvement of noncoding RNAs in coordinating *var* gene regulation; however, these hypotheses remain controversial.

Here, we explore these hypothesized models using transgenic parasite lines in which *var* gene expression can be manipulated using drug selection, and in which specific *var* loci have been tagged for visualization by fluorescent *in situ* hybridization (FISH). The data show that cooperative interactions between two promoters found in each gene are required for both silencing and mutually exclusive expression. Disruption of these interactions results in simultaneous expression of several *var* genes in the same parasite, thus definitively showing the necessity of cooperative regulatory elements in *var* gene regulation. Furthermore, heterologous promoters can act in place of *var* intron promoter elements for cooperative regulation, providing information on the silencing mechanism. Simultaneously active *var* genes colocalize within the nucleus, providing strong evidence for a postulated subnuclear expression site, but also suggesting that it is capable of accommodating several transcriptionally active *var* promoters.

RESULTS AND DISCUSSION

A second promoter is required for *var* gene silencing

Several previous studies have implicated the intron found in all *var* genes in the epigenetic silencing of *var* gene expression (Deitsch *et al*, 2001a; Calderwood *et al*, 2003; Gannoun-Zaki *et al*, 2005; Frank *et al*, 2006). One possible mechanism for this silencing effect is the classic model of a repressor-binding site existing within the intron that is bound by proteins either preventing transcription complexes from accessing the upstream *var* promoter, or recruiting chromatin modifiers, which results in the assembly of a silent chromatin state. However, it was also shown that *var* introns have independent promoter activity and that deleting the part of the intron containing this promoter disrupts the silencing properties of the intron (Calderwood *et al*, 2003). These observations indicate a possible alternative model for the mechanism that underlies the silencing effect of the intron—namely, that it is the promoter activity itself rather than the DNA sequence that is responsible for the ability of the intron to silence a *var* gene. To test this hypothesis, we created a series of plasmid constructs in which we placed any one of several, unrelated *P. falciparum* gene promoters in close proximity to a *var* promoter that drives expression of a firefly *luciferase* reporter gene. We found that the *P. falciparum* heat-shock protein 86 (*hsp86*) promoter and promoters for genes such as histidine-rich protein 2 (*hrp2*) and *ribosomal RNA* somehow interact, either directly or indirectly, with a *var* upstream promoter, resulting in cooperative silencing similar to that observed with the *var* intron (Fig 1). Moreover, as previously observed for cooperative interactions with a *var* intron promoter (Deitsch *et al*, 2001a), silencing in all cases was S-phase-dependent, strengthening the conclusion that it is the promoter activity of these sequences that contributes to the epigenetic processes responsible for silencing.

The ability of promoter activity, rather than simple DNA sequence elements, to participate in epigenetic silencing is not

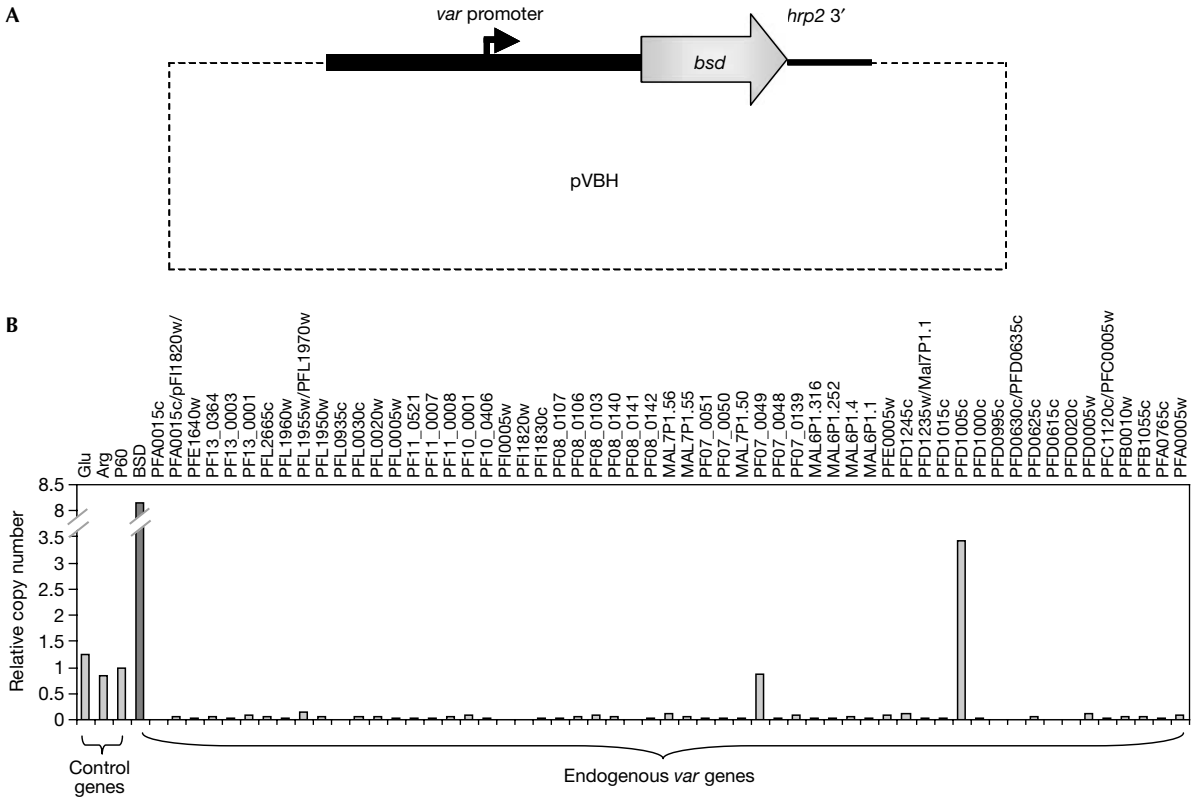

Fig 2 | Simultaneous expression of more than one *var* promoter in transfected parasites. (**A**) Schematic map of the plasmid pVBH. (**B**) Levels of *var* gene expression detected by quantitative reverse transcription–PCR of complementary DNA obtained from transfected parasites. The columns show messenger RNA levels of each *var* gene in the parasite genome, the episomal *bsd* gene and three housekeeping control genes. RNA was extracted from a population in which one *var* gene (PFD1005c) was predominantly expressed above the others. Note that *bsd* is transcribed at a relatively high level, reflecting its expression from episomes by all parasites within the population.

without precedent. Replacement of promoters in the X-inactivation centre of mammalian X chromosomes with heterologous promoters from elsewhere in the genome does not disrupt silencing or counting, but can influence choice (Stavropoulos *et al*, 2001). Our data also provide a possible explanation for *var* upstream promoter silencing in the absence of a *var* intron that was recently reported (Voss *et al*, 2006). The plasmids used in this study also carried an *hsp86* promoter (for expression of a drug-resistant cassette) in the neighbourhood of the *var* upstream promoter. As a consequence, the plasmids were subjected to cooperative silencing from promoter–promoter interactions similar to that observed for the constructs in the experiments shown in Fig 1, thus potentially rendering the intron redundant and unnecessary for silencing.

Cooperative interactions and *var* gene recognition

An additional question for understanding *P. falciparum* antigenic variation concerns how *var* genes are recognized by the mechanism that controls mutually exclusive expression. Previous work has shown that this mechanism normally restricts expression to a single *var* upstream promoter at any point in time, regardless of whether it is a transgenic promoter on an episome or an endogenous promoter in the chromosome (Dzikowski *et al*, 2006; Voss *et al*, 2006). In addition, analysis of concatameric episomes suggested that strict one-to-one pairing between *var* upstream and intron promoters is required not only for silencing, but also for *var* gene recognition and mutually exclusive expression (Frank *et al*, 2006). This is in contrast to a model recently proposed by Voss *et al* (2006), who reported that parasites are able to express only a single *var* upstream promoter at a time, regardless of intron pairing. As discussed above, however, the *var* promoters examined in those experiments might have been interacting with a neighbouring *hsp86* promoter, and thus might not in fact have been 'unpaired'.

In an attempt to determine definitively whether a 'free' *var* promoter is indeed recognized by the *P. falciparum* allelic exclusion mechanism, we carried out experiments using a plasmid (pVBH; Fig 2A) in which an upstream *var* promoter was truly isolated from other sequences that could influence its transcriptional activity. Because the blasticidin S deaminase (*bsd*) cassette in pVBH is driven by this *var* promoter, blasticidin selection of transfected parasites ensured that all parasites in the drug-selected population contained an active episomal *var* promoter. Analysis of transcription of the entire *var* gene family in these transfected parasites detected active transcription of the episomal *bsd* gene along with two endogenous *var* genes (PF07-0049 and PFD1005c; Fig 2B), confirming that the 'unpaired' episomal *var* promoter was not recognized by the mechanism controlling mutually exclusive

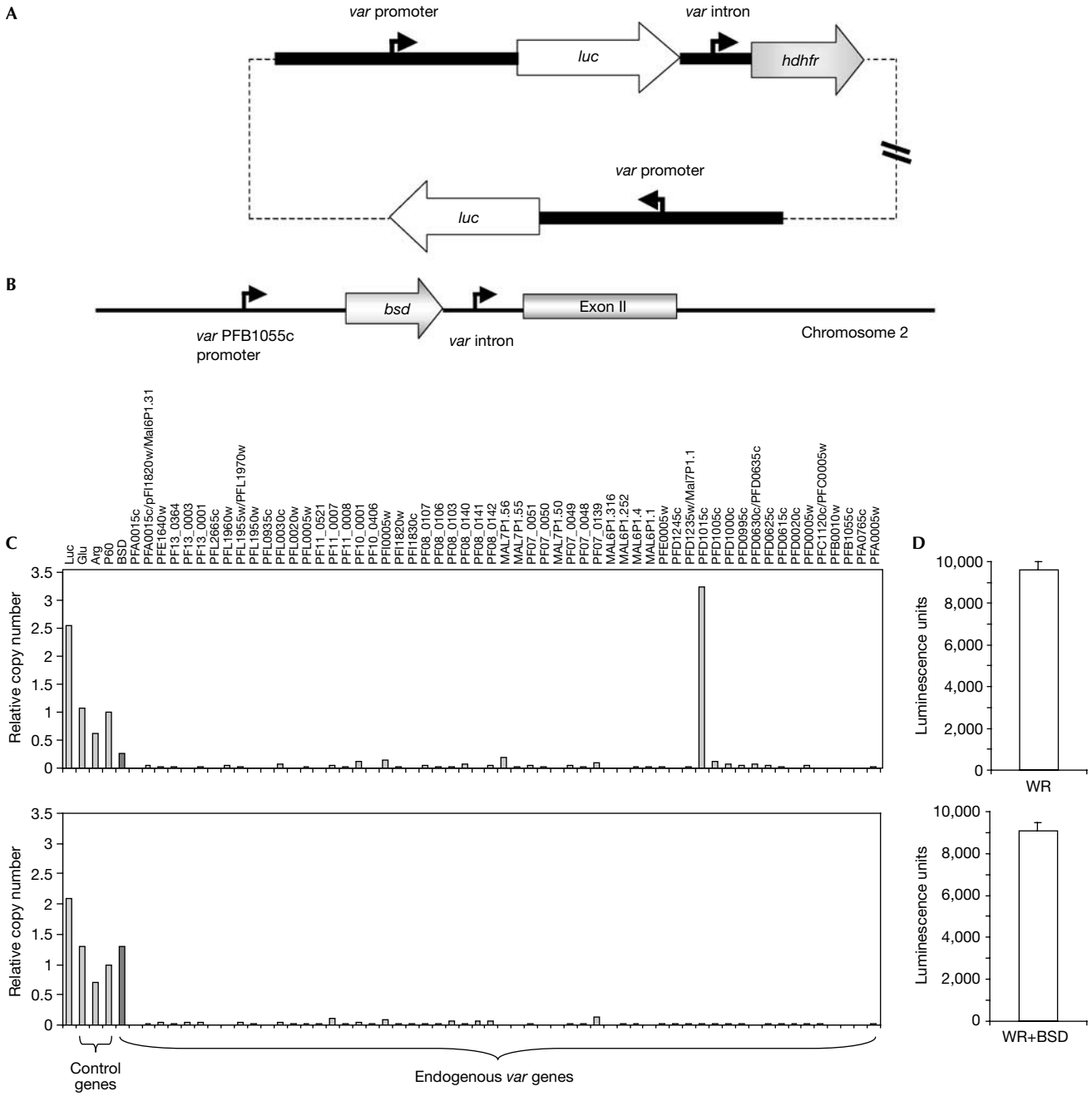

Fig 3 | Expression of *luciferase*, *bsd* and the *var* gene family in transfected parasite lines. (**A**) Schematic map of pVLH/IDH-FP. (**B**) Schematic map of the *var* gene PFB1055c in the transgenic parasite line B12E3. (**C**) Results confirming simultaneous transcription of two *var* promoters in stably transfected parasite lines. Upper panel: B12E3 stably transfected with pVLH/IDH-FP and grown in the absence of blasticidin. Both the endogenous *var* gene PFD1015c and *luciferase* are highly expressed, whereas all other *var* genes are silent. Lower panel: the same parasite line, grown for several weeks under blasticidin pressure. Expression switched to the recombinant *var* (PFB1055c) containing the *bsd*-resistant gene, which was then transcribed in a mutually exclusive manner. However, the expression level of the *luciferase* gene being driven by an 'unpaired' *var* promoter remains unchanged. (**D**) Simultaneous protein expression by two *var* promoters in stably transfected parasite lines. Luciferase is highly expressed in transfected parasites grown with (lower panel) or without (upper panel) blasticidin pressure. Stable episomes were maintained using WR99210 (WR) pressure to select for expression of the human *dihydrofolate reductase* gene (*hdhfr*).

expression. Because individual parasites would not survive as drug-sensitive forms in the presence of blasticidin, these results argue strongly for joint expression of *bsd* under the control of the 'unpaired' episomal *var* promoter together with expression of an endogenous *var* gene in each parasite. Detection of transcripts from two endogenous *var* genes in the transfectants is consistent

with *var* gene switching and the development of subpopulations carrying two different forms of PfEMP1.

A possible criticism of the above experiments is that the *var* promoter in pVBH had been selected by drug pressure to be active, so its default state of transcriptional activity thus might not have been accurately observed. To address this possibility, we carried out additional experiments with a plasmid containing two *var* upstream promoters, one paired with an intron and the other not paired and therefore constitutively active (pVLH/IDH-FP; Fig 3A; Frank *et al*, 2006). In transfectants carrying this plasmid, it was therefore possible to measure *luciferase* messenger RNA levels from an unpaired *var* promoter that had not been under direct selection. This plasmid was transfected into parasites that had been previously engineered by allelic replacement to carry a *bsd* cassete at *var* locus PFB1055c (Fig 3B; Dzikowski *et al*, 2006). The recombinant *var* promoter driving *bsd* expression at this locus is paired with the endogenous intron, and previous work has shown that it is recognized and subjected to mutually exclusive expression (Dzikowski *et al*, 2006).

Results from the pVLH/IDH-FP transfectants free of blasticidin pressure showed both active *luciferase* transcription from the constitutively active plasmid promoter and high levels of transcription from the endogenous *var* gene PFD1015c (Fig 3C). This suggests that the unpaired promoter was not recognized by the exclusive control mechanism and that more than one *var* promoter was transcribed in individual parasites. When the pVLH/IDH-FP-transfected parasites were subjected to blasticidin selection, only those parasites that switched to *bsd* expression under the PFB1055c promoter were able to survive. In these parasites, expression of PFD1015c and all other chromosomal *var* genes were silenced (Fig 3C), indicating that the endogenous *var* promoter driving *bsd* expression that is paired with an intron is recognized and expressed in a mutually exclusive manner. Nevertheless, these parasites continued to express high levels of luciferase (Fig 3D).

To validate further that both *var* promoters were, in fact, simultaneously active in the same cell, RNA-FISH was carried out to detect active transcription of both *bsd* and *luciferase* in individual nuclei. These experiments detected both *var* promoter-driven transcripts in 30 out of 30 nuclei examined (Fig 4A, lower panel), confirming that a *var* promoter that is separated from its intron (or other paired promoter activity) remains active in the presence of an actively transcribed endogenous *var* locus, and thus is not recognized by the exclusive expression mechanism. Interestingly, in the cells examined, both *var* promoter-driven transcripts frequently localized within the same region of the nucleus, leading us to investigate the possible existence of a *var*-specific expression site.

Detection of a *var*-specific subnuclear expression site

Investigations into the structure of the *P. falciparum* nucleus have identified a region of uncondensed euchromatin in the largely heterochromatic nuclear periphery (Ralph *et al*, 2005). This subnuclear region has been suggested to function as a specific expression site for *var* promoters, and a model for mutually exclusive expression proposes that this site can accommodate only one *var* promoter at a time, similar to a model proposed for the mutually exclusive expression of *vsg* genes in African trypanosomes (Navarro & Gull, 2001). Furthermore, some recent publications have reported that *var* genes move to a different position in the nucleus on transcriptional activation (Duraisingh *et al*, 2005; Ralph *et al*, 2005; Voss *et al*, 2006). However, it has not yet been determined whether active *var* genes move to a specific site, or whether they simply move to one of several transcriptionally competent regions of the nucleus. We were therefore interested to localize *var* transcription in our transgenic parasite lines containing two simultaneously active *var* promoters.

Using DNA-FISH and probes specific to the pVLH/IDH-FP episome or to the recombinant chromosomal gene expressing *bsd*, we found that the constitutively active, unpaired episomal *var* promoter colocalized with the active PFB1055c-driven *bsd* gene in greater than 90% of the transfectants selected by blasticidin (Fig 4B,C). By contrast, less than 10% of cells showed colocalization in transfected parasite populations without blasticidin pressure and primarily expressing the endogenous *var* gene PFD1015c. Removal of WR99210 pressure resulted in rapid shedding of the plasmid (confirmed by quantitative PCR) and loss of luciferase signal, indicating that in all cases the pVLH/IDH-FP plasmid had not integrated into the genome.

The level of colocalization between the simultaneously active *var* promoters detected here (>90%) was higher than previously reported for the colocalization of an active *var* promoter with the promoter of an unrelated, constitutively active gene (~60%; Duraisingh *et al*, 2005). We also found that an active *var* gene frequently colocalized with the active, unrelated gene PFF1125c; however, the rate of colocalization was lower than that observed for two active *var* genes (supplementary Fig S1 online). This might indicate that the nucleus of the parasite contains a limited number of sites of transcriptional activity, and thus transcriptionally active genes, regardless of promoter type, tend to colocalize frequently in these subnuclear regions. However, *var* promoters seem to require more strict localization into a specific subnuclear site, thus resulting in the near-complete colocalization of the two active *var* promoters detected in this study. This might indicate the presence in the nucleus of a localized, *var*-specific factor necessary for activation of *var* gene transcription, although this factor seems to be able to actively transcribe at least two *var* promoters simultaneously, suggesting that restricted accommodative capacity of the expression site alone does not explain strict mutually exclusive *var* gene expression. Furthermore, the two active *var* promoters in the FISH images frequently seemed to be immediately adjacent to one another, rather than completely overlapping, suggesting a relatively large subnuclear region (see supplementary Fig S2 online for images). Interestingly, a silent *var* promoter colocalized with an active unrelated promoter approximately 25% of the time (supplementary Fig S1 online), whereas active and silent *var* promoters colocalized in only 10% of cases, indicating that there might be a mechanism that actively separates active and silent *var* genes. Further work into the structure and function of *Plasmodium* nuclear subdomains will provide more information on this potentially important aspect of *var* gene regulation.

METHODS

Parasite culture and transfection. All experiments used the *P. falciparum* NF54 line cultivated by standard methods (Trager & Jensen, 1976). Parasites were transfected by spontaneous uptake of DNA in plasmid-loaded red blood cells as described previously

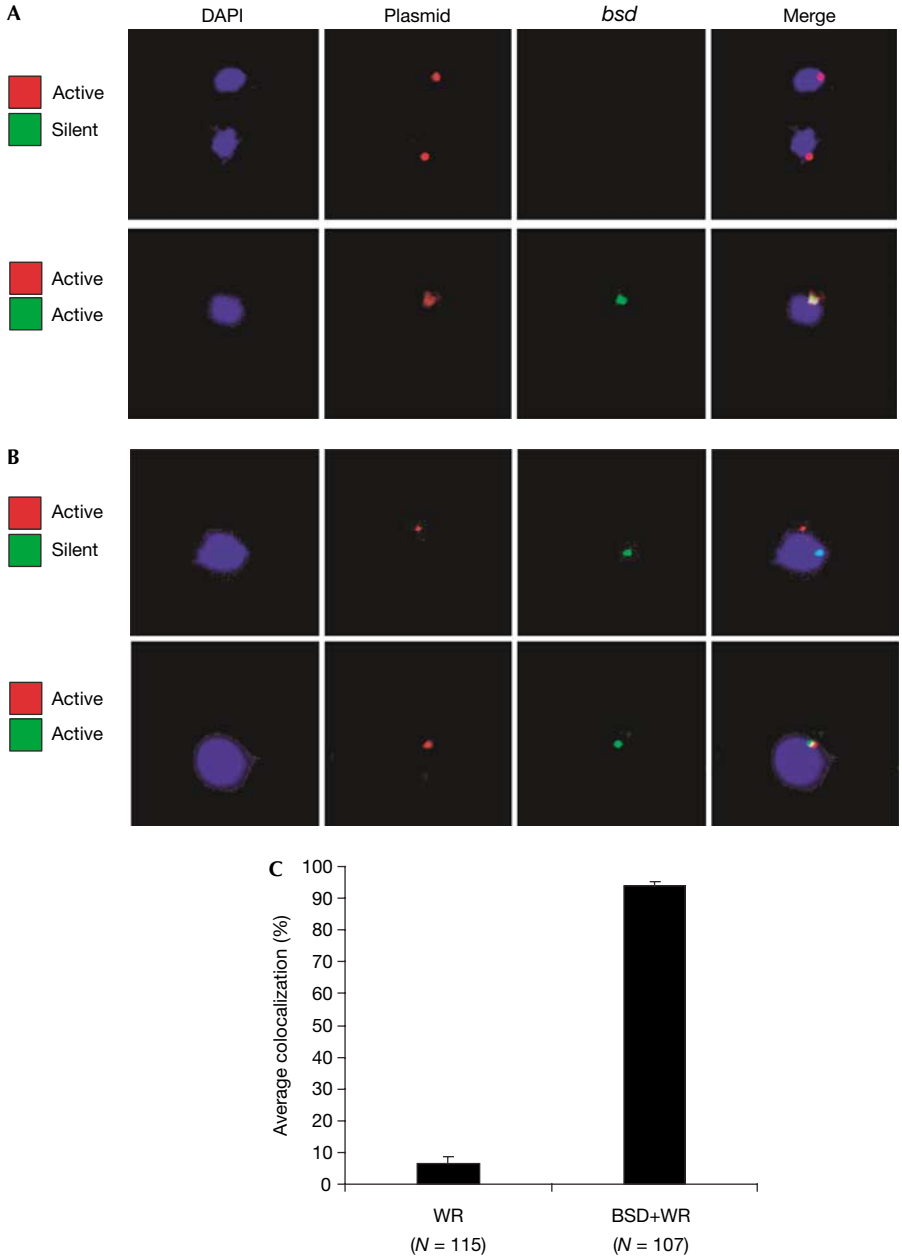

Fig 4 | Nuclear positioning of two *var* loci in different activation states. Nuclei are stained with DAPI (blue). (**A**) RNA-FISH. Upper panel: nuclear positioning of messenger RNA actively transcribed from the *luciferase* gene (red) on the plasmid pVLH/IDH-FP in cells in which the chromosomal *bsd* cassette is silent. Lower panel: nuclear positioning of mRNA of both *luciferase* (red) and the endogenous *bsd* cassette (green) when they are both actively expressed. (**B**) DNA-FISH. Upper panel: nuclear positioning of actively transcribed pVLH/IDH-FP episomes (red) and of a silent *bsd* cassette under the control of a chromosomal *var* promoter (green). Lower panel: nuclear positioning when both *luciferase* (red) carried on the pVLH/IDH-FP episomes and the endogenous *bsd* cassette (green) are actively expressed. (**C**) Quantification of colocalization shown in (**B**). Error bars show standard deviations of multiple, independent counts. DAPI, 4',6-diamidino-2-phenylindole; FISH, fluorescent *in situ* hybridization; WR, WR99210 selection.

(Deitsch *et al*, 2001b). Transfected parasites were selected in 4 nM WR99210 and/or 2 μg/ml blasticidin as appropriate.

DNA constructs. The plasmids pVLH, pVLH/*int*, and pVLH/IDH-FP were described previously (Deitsch *et al*, 2001a; Dzikowski *et al*, 2006; Frank *et al*, 2006). The other plasmid constructs were derived from these as described in detail in the supplementary information online.

Assays of *var* transcription by real-time reverse transcription–PCR. RNA extraction, production of complementary DNA and analysis of expression of the *var* gene family were carried out as described previously (Dzikowski *et al*, 2006; Frank *et al*, 2006).

Fluorescent *in situ* hybridization. DNA-FISH was carried out on ring-stage parasites as described previously (Freitas-Junior *et al*, 2000) with slight modifications (see the supplementary

information online). Images of nuclei were collected, randomly coded and scored independently by three individuals. RNA-FISH was carried out as described previously (Thompson, 2002) with slight modifications (see the supplementary information online). **Supplementary information** is available at *EMBO reports* online (http://www.emboreports.org).

ACKNOWLEDGEMENTS

We thank O. Dzikowski and D. Raskolnikov for their help in counting and scoring colocalization in the FISH experiments. This work was supported by National Institutes of Health (NIH) grant AI 52390 and a grant from the Ellison Medical Foundation. The Department of Microbiology and Immunology at Weill Medical College of Cornell University acknowledges the support of the William Randolph Hearst Foundation. T.E.W. is supported by the Intramural Research Program of the NIH, NIAID. K.W.D. is a Stavros S. Niarchos Scholar.

REFERENCES

Arney KL, Erhardt S, Drewell RA, Surani MA (2001) Epigenetic reprogramming of the genome—from the germ line to the embryo and back again. *Int J Dev Biol* **45:** 533–540

Calderwood MS, Gannoun-Zaki L, Wellems TE, Deitsch KW (2003) *Plasmodium falciparum* var genes are regulated by two regions with separate promoters, one upstream of the coding region and a second within the intron. *J Biol Chem* **278:** 34125–34132

Chaves I, Zomerdijk J, Dirks-Mulder A, Dirks RW, Raap AK, Borst P (1998) Subnuclear localization of the active variant surface glycoprotein gene expression site in *Trypanosoma brucei*. *Proc Natl Acad Sci USA* **95:** 12328–12333

Corcoran AE (2005) Immunoglobulin locus silencing and allelic exclusion. *Semin Immunol* **17:** 141–154

Deitsch KW, Calderwood MS, Wellems TE (2001a) Malaria. Cooperative silencing elements in var genes. *Nature* **412:** 875–876

Deitsch KW, Driskill CL, Wellems TE (2001b) Transformation of malaria parasites by the spontaneous uptake and expression of DNA from human erythrocytes. *Nucleic Acids Res* **29:** 850–853

Duraisingh MT, Voss TS, Marty AJ, Duffy MF, Good RT, Thompson JK, Freitas-Junior LH, Scherf A, Crabb BS, Cowman AF (2005) Heterochromatin silencing and locus repositioning linked to regulation of virulence genes in *Plasmodium falciparum*. *Cell* **121:** 13–24

Dzikowski R, Frank M, Deitsch K (2006) Mutually exclusive expression of virulence genes by malaria parasites is regulated independently of antigen production. *PLoS Pathog* **2:** e22

Frank M, Dzikowski R, Constantini D, Amulic B, Burdougo E, Deitsch K (2006) Strict pairing of var promoters and introns is required for var gene silencing in the malaria parasite *Plasmodium falciparum*. *J Biol Chem* **281:** 9942–9952

Freitas-Junior LH, Bottius E, Pirrit LA, Deitsch KW, Scheidig C, Guinet F, Nehrbass U, Wellems TE, Scherf A (2000) Frequent ectopic recombination of virulence factor genes in telomeric chromosome clusters of *P. falciparum*. *Nature* **407:** 1018–1022

Gannoun-Zaki L, Jost A, Mu JB, Deitsch KW, Wellems TE (2005) A silenced *Plasmodium falciparum* var promoter can be activated *in vivo* through spontaneous deletion of a silencing element in the intron. *Eukaryotic Cell* **4:** 490–492

Kyes S, Horrocks P, Newbold C (2001) Antigenic variation at the infected red cell surface in malaria. *Annu Rev Microbiol* **55:** 673–707

Merrick CJ, Duraisingh MT (2006) Heterochromatin-mediated control of virulence gene expression. *Mol Microbiol* **62:** 612–620

Navarro M, Gull K (2001) A pol I transcriptional body associated with VSG mono-allelic expression in *Trypanosoma brucei*. *Nature* **414:** 759–763

Okamoto I, Otte AP, Allis CD, Reinberg D, Heard E (2004) Epigenetic dynamics of imprinted X inactivation during early mouse development. *Science* **303:** 644–649

Ralph SA, Scheidig-Benatar C, Scherf A (2005) Antigenic variation in *Plasmodium falciparum* is associated with movement of var loci between subnuclear locations. *Proc Natl Acad Sci USA* **102:** 5414–5419

Scherf A, Hernandez-Rivas R, Buffet P, Bottius E, Benatar C, Pouvelle B, Gysin J, Lanzer M (1998) Antigenic variation in malaria: *in situ* switching, relaxed and mutually exclusive transcription of *var* genes during intra-erythrocytic development in *Plasmodium falciparum*. *EMBO J* **17:** 5418–5426

Serizawa S, Miyamichi K, Sakano H (2004) One neuron–one receptor rule in the mouse olfactory system. *Trends Genet* **20:** 648–653

Stavropoulos N, Lu N, Lee JT (2001) A functional role for Tsix transcription in blocking Xist RNA accumulation but not in X-chromosome choice. *Proc Natl Acad Sci USA* **98:** 10232–10237

Thompson J (2002) *In situ* detection of RNA in blood- and mosquito-stage malaria parasites. *Methods Mol Med* **72:** 225–233

Trager W, Jensen JB (1976) Human malaria parasites in continuous culture. *Science* **193:** 673–675

Voss TS, Healer J, Marty AJ, Duffy MF, Thompson JK, Beeson JG, Reeder JC, Crabb BS, Cowman AF (2006) A var gene promoter controls allelic exclusion of virulence genes in *Plasmodium falciparum* malaria. *Nature* **439:** 1004–1008