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A well-conserved *Plasmodium falciparum var* gene shows an unusual stage-specific transcript pattern

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Summary

The *var* multicopy gene family encodes *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) variant antigens, which, through their ability to adhere to a variety of host receptors, are thought to be important virulence factors. The predominant expression of a single cytoadherent PfEMP1 type on an infected red blood cell, and the switching between different PfEMP1 types to evade host protective antibody responses, are processes thought to be controlled at the transcriptional level. Contradictory data have been published on the timing of *var* gene transcription. Reverse transcription-polymerase chain reaction (RT-PCR) data suggested that transcription of the predominant *var* gene occurs in the later (pigmented trophozoite) stages, whereas Northern blot data indicated such transcripts only in early (ring) stages. We investigated this discrepancy by Northern blot, with probes covering a diverse *var* gene repertoire. We confirm that almost all *var* transcript types were detected only in ring stages. However, one type, the well-conserved *varCSA* transcript, was present constitutively in different laboratory parasites and does not appear to undergo antigenic variation. Although *varCSA* has been shown to encode a chondroitin sulphate A (CSA)-binding PfEMP1, we find that the presence of full-length *varCSA* transcripts does not correlate with the CSA-binding phenotype.

Introduction

Plasmodium falciparum is responsible for nearly all malaria-specific mortality and for a high proportion of overall malaria morbidity. The particular virulence of this species is partly attributed to modifications to the host erythrocyte membrane during asexual infection. Parasite proteins inserted into the infected red blood cell membrane mediate adhesion to a variety of host receptors on vascular endothelium, uninfected red blood cells, placental syncytiotrophoblast cells, platelets and dendritic cells (reviewed by Kyes *et al*., 2001). The downstream effects of these interactions are thought to underlie much of the pathogenesis of severe disease. One major group of parasite proteins involved is a variant antigen family, collectively termed *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) and encoded by the *var* multicopy gene family (Baruch *et al*., 1995; Smith *et al*., 1995; Su *et al*., 1995). PfEMP1 proteins are also targets of host protective antibodies (Bull *et al*., 1998), but antigenic variation between PfEMP1-types leads to evasion of the host immune response during chronic infection.

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Placental malaria is a special case of infected red blood cell adhesion, causing maternal anaemia and low birth-weight in the first pregnancies of women who already have partial antimalarial immunity (Brabin, 1983). In primigravidae, infected red blood cells can concentrate in the placenta, adhering to syncytiotrophoblast cells via chondroitin sulphate A (CSA; Fried and Duffy, 1996) or hyaluronic acid (Beeson *et al.*, 2000). In subsequent pregnancies, a relative resistance develops, possibly caused by specific antibodies recognizing a restricted repertoire of antigens on the surface of infected red blood cells in the placenta (Fried *et al.*, 1998; Buffet *et al.*, 1999; Ricke *et al.*, 2000; Lekana Douki *et al.*, 2002). This situation contrasts with that in infections of non-pregnant hosts, where the repertoire of variant antigens expressed is extremely large (e.g. Marsh *et al.*, 1986; Aguiar *et al.*, 1992). It has thus become an important goal to define the subset of variant antigens expressed in the placenta, as they may form the basis of a specific vaccine against placental malaria.

Although there is evidence for non-PfEMP1-mediated ring-stage parasite adhesion that correlates with CSA binding at the pigmented trophozoite stage (Pouvelle *et al.*, 2000), the *var* gene family is the best studied variant antigen responsible for adhesive events. Each haploid parasite genome contains ≈60 *var* genes (Gardner *et al.*, 2002), which are highly diverse in sequence when compared within a single genome and between isolates (Freitas-Junior *et al.*, 2000; Taylor *et al.*, 2000; Fowler *et al.*, 2002). The highly variable 5′ exon (3.5–10 kbp exon 1) codes for the extracellular portion of the protein plus a transmembrane segment, and the semi-conserved 3′ exon (1–1.5kb exon 2) codes for a cytoplasmic region. The genes for two different CSA-binding PfEMP1 variants have been identified in IT lineage laboratory parasites (equivalent to FCR3, Robson *et al.*, 1992; *var*-CS2, Reeder *et al.*, 1999; and *FCR3.varCSA*, Buffet *et al.*, 1999). Although *var-CS2* appears to be unique to the IT/FCR3 lineage, *FCR3.varCSA* is well conserved between isolates (Rowe *et al.*, 2002; Salanti *et al.*, 2002) and is referred to here as *varCSA*. Its unusually high degree of conservation between isolates makes *varCSA* a strong candidate for an antiplacental malaria vaccine. However, transcripts for *varCSA* are found in both placental and non-placental isolates (Fried and Duffy, 2002), raising some doubt as to how strictly *varCSA* is limited to placental isolates.

Because of the importance of PfEMP1 in pathogenesis and immune evasion, much effort has been expended on determining the molecular mechanisms controlling *var* gene expression. However, investigations on the timing of transcription within the cell cycle and on the range of *var* genes transcribed within any one parasite population have led to contradictory results. Reverse transcription-polymerase chain reaction (RT-PCR) and nuclear run-on data (Chen *et al.*, 1998a; Scherf *et al.*, 1998) suggested that many different *var* gene variants are transcribed in early (10 h after invasion; ring) stages of the asexual life cycle, and exclusive transcription of the gene encoding the single cytoadherent variant occurs in later (24 h after invasion; pigmented trophozoite) stages. Our Northern blot data (Smith *et al.*, 1998; Kyes *et al.*, 2000) indicated that full-length transcripts encoding the cytoadherent variant are present only in ring (3–18 h after invasion) stages, and correlated well with the stage specificity of PfEMP1-mediated binding phenotypes, starting at about 16 h after invasion (Gardner *et al.*, 1996).

We tried unsuccessfully to reproduce the RT-PCR results described above in 3D7, the laboratory parasite used for the Malaria Genome Sequencing Project, but recent developments have allowed us to reinterpret our data. We reported previously a comparison of 3D7 ring versus pigmented trophozoite *var* transcripts (Taylor *et al.*, 2000), using RT-PCR to amplify variant-specific sequence tags representing a short fragment within the most conserved region of exon 1 [Duffy-binding-like domain alpha (DBLα); for a description, see Smith *et al.*, 2000]. We found multiple *var* types at low frequency in both stages, but a much

higher frequency of one type (3D7AFBR4; accession no. AF133860) was seen in pigmented trophozoites. This type did not correlate with the major full-length *var* transcript detected on Northern blots. We now know through data from the Malaria Genome Sequencing Project that 3D7AFBR4 happens to be the variant-specific tag for 3D7 *varCSA*, and also that the 3D7 *varCSA* is truncated, lacking exon 2 (*3D7chr5var*; Rowe *et al.*, 2002). In this case, the predominance of a single transcript type detected by RT-PCR in pigmented trophozoites clearly cannot relate to PfEMP1 expression, as the 3D7 *varCSA* is a pseudogene.

We thus resolved to re-examine the timing of *var* transcription, paying particular attention to the *varCSA* gene.

Results

Relative and absolute levels of full-length *var* transcripts

To investigate the discrepancy between Northern blot and RT-PCR data regarding the timing of *var* gene transcription, we first re-examined the design of our published time course Northern blots (Kyes *et al.*, 2000). These data compared equivalent amounts of total RNA, showing relative transcript levels at each time point. However, accumulated RNA levels increase at least fourfold from rings to pigmented trophozoite stages (Newbold *et al.*, 1982; data not shown), so this approach may have given a misleading picture of absolute *var* transcript levels over time. Therefore, we repeated the time course analysis, but prepared RNA from known numbers of cells, at 4 h intervals, from a single culture of tightly synchronized, unselected A4 parasites ($A4^{ICAM\text{-}U}$). Blots comparing total RNA from equivalent numbers of cells at each time point were hybridized at low stringency with the complex *var* exon 2 probe. Confirming our previous reports (Kyes *et al.*, 2000), full-length *var* transcripts were only detected in ring stages (8–20 h after invasion), not in pigmented trophozoites (after 20 h; Fig. 1A). In stages 24 h after invasion and later, an ≈3 kb transcript is presumed to represent transcripts of Pf60 (Bonnefoy *et al.*, 1997), another multigene family with high similarity to *var* exon2, or aberrant exon 2 transcripts (Su *et al.*, 1995).

Complex *var* exon 2 is not a universal probe for *var* genes

The discrepancy in observed timing of *var* gene transcription was not resolved by comparing absolute *var* transcript levels (per equivalent number of cells) with relative levels (per equivalent amounts of RNA) on Northern blots, so we tested whether the complex *var* exon 2 probe detects all *var* genes. In analysing *var* gene transcripts in the IT lineage, we had already noticed that *R29var1* (Rowe *et al.*, 1997) did not hybridize with a ‘generic’ probe, varC, which was based on the exon 2 sequence and presumed to be semi-conserved between all *var* gene types (Rubio *et al.*, 1996). Indeed, sequence analysis showed that the exon 2 of *R29var1* cannot be amplified with the varC primers. Therefore, for a more comprehensive detection of *var* transcripts, we had devised the ‘complex *var* exon 2’ probe, by mixing varC with a cloned *R29var1* exon 2 fragment. However, of the published IT/FCR3 lineage *var* genes, the exon 2 of *FCR3.varCSA* is also unique. To be certain that we could detect this particular *var* transcript, and any other possible *var* genes with related exon 2 types, we designed primers to amplify *FCR3.varCSA* exon 2 and hybridized the labelled fragment to the $A4^{ICAM\text{-}U}$ time course blots (Fig. 1B). Two distinct transcript patterns were detected. In ring-stage parasites (8–16 h after invasion), barely detectable transcripts of 9–13 kb were present, similar to those detected by the complex *var* exon 2 probe. In late rings and pigmented trophozoites, however, a single large transcript of ≈15 kb was present at high levels (from 20 h after invasion). The same transcript was present only at very low levels during ring stages. Again, the 3 kb transcript in pigmented trophozoites probably represents Pf60/aberrant exon 2. Thus, the conflict in published results on *var* transcriptional timing

could lie in the specific type of *var* gene being investigated, as distinguished by different exon 2 probes.

The single 15 kb *var* transcript detected in all asexual stages is identical to *FCR3.varCSA*

We also characterized the 15 kb transcript detected by the *varCSA* exon 2 probe. It corresponds to the relatively large size of *FCR3.varCSA* (Buffet *et al.*, 1999), and its identity was confirmed by high-stringency hybridization of the same blot with various probes specific for *FCR3.varCSA* exon 1 (Fig. 1C; DBL7 is representative; DBL4 + DBL5 probe was also tested; data not shown). These probes only detected the 15 kb band, and it was present in all asexual stages. The pigmented trophozoite stages appear to have much higher levels of transcripts per infected red cell than ring stages. The 3 kb (Pf60/aberrant exon 2) band is not detected with this exon 1 probe, as expected.

The unique timing of *varCSA* transcript detection occurs in several different laboratory parasites

The *varCSA* gene transcript profile that we observed in this time course could be a unique feature of the $A4^{ICAM-U}$ parasite, so we compared this parasite with the genetically related $FCR3^{CSA-U}$ and with unrelated TM180. On blots loaded with equivalent levels of RNA per lane and probed at high stringency with *varCSA* DBL4 + DBL5, a similar pattern is found in $A4^{ICAM-U}$ and $FCR3^{CSA-U}$ parasites. The 15 kb *varCSA* transcript is present at low levels in early to mid-rings and at much higher levels in late rings/pigmented trophozoites (Fig. 1E and F). With slightly less stringent washing conditions (60°C instead of 65°C to accommodate differences in levels of sequence similarity between TM180 and FCR3 *varCSA* genes), TM180 parasites have two transcripts, a 12 kb band detected only in rings and the 15 kb band detected in all stages (Fig. 1G). The 12 kb transcripts are ring stage specific, detected with the complex *var* exon 2 probe from 6 to 18 h after invasion, but not from 24 to 48 h after invasion (Fig. 1H).

We compared two further distinct parasite genotypes, 3D7 and HB3, for their *var* gene transcription pattern. Mid- to late-ring and pigmented trophozoite stage RNA samples were loaded with equivalent amounts of total RNA per lane, as well as equivalent cell numbers per lane (data not shown). As before, the complex *var* exon 2 probe detects transcripts of varying sizes, restricted to ring stages in all parasites (Fig. 2A). This probe detects no full-length *var* transcripts in pigmented trophozoite stages, regardless of how much RNA was loaded (data not shown). The *varCSA* exon 2 probe (Fig. 2B) detects multiple *var* transcripts in ring stages for the three unrelated parasites, and a 15 kb transcript in $A4^{ICAM-U}$ and HB3, in both rings and pigmented trophozoites. Hybridization with the *varCSA* DBL7 probe detects only a 15 kb band in $A4^{ICAM-U}$ and HB3, matching the timing of the 15 kb band detected by the *varCSA* exon 2 probe (Fig. 2C). No full-length transcript for the *varCSA* gene was detected with either probe in 3D7, at either stage, as expected because of the truncation of this gene in 3D7 (Rowe *et al.*, 2002).

A4 and FCR3 parasites with ICAM-1 or CSA binding phenotypes contain *varCSA* transcripts in ring and pigmented trophozoite stages

The $A4^{ICAM-U}$, HB3 and TM180 parasites had never been selected for CSA binding. The A4 clone had originally been selected for intercellular adhesion molecule-1 (ICAM-1) binding, but this particular culture had not been selected for any binding phenotype for at least 20 cycles before RNA extraction. We therefore compared RNA from related parasites before and after selection for specific phenotypes. We compared A4 with low ($A4^{ICAM-U}$) and high ($A4^{ICAM-1C1}$) ICAM-1 binding phenotypes, and $FCR3^{CSA}$ before ($FCR3^{CSA-U}$) and after ($FCR3^{CSA+}$) selection on CSA. The complex *var* exon 2 probe (Fig. 3A) detected two bands in $A4^{ICAM-U}$ and $A4^{ICAM-IC1}$. The large (13 kb) band corresponds to previously

reported ICAM-1-binding PfEMP1 transcript *A4var* (Smith *et al.*, 1998). A diffuse band at around 9 kb probably represents multiple *var* transcripts of a similar size range. The 13 kb band was enriched by ≈13% (relative to the diffuse band) in the higher ICAM-1-binding parasite, $A4^{ICAM\text{-}IC1}$ (as judged by densitometry), corresponding well with the modest increase in ICAM-1 binding. The same probe detected two bands in $FCR3^{CSA\text{-}U}$ (9.5 kb and barely visible 14 kb), and two bands (equal intensities of 9.5 kb and 14 kb) in $FCR3^{CSA+}$. The *varCSA* DBL7 probe (Fig. 3B) detects a 15 kb transcript in both rings and pigmented trophozoites of all parasites, regardless of the binding phenotype.

Parasites containing full-length transcripts for the *varCSA* gene do not necessarily bind to CSA

All parasites tested, except for 3D7, which has a truncated version of the gene, seemed to contain the *varCSA*-type transcripts either in all asexual stages or mainly in pigmented trophozoite stages. It remained possible that all parasites bind CSA to some extent, thus explaining the apparent universal presence of the conserved *varCSA* transcripts. We performed standard infected red blood cell binding assays to ICAM-1, CSA and 1% BSA spotted on plastic dishes (Fig. 3C). Several parasites bound to ICAM-1 significantly [>1000 infected RBC mm^{-2}; TM180 (not shown), $A4^{ICAM\text{-}U}$, $A4^{ICAM\text{-}1C1}$]. Three had low binding to ICAM-1 [50–600 infected RBC mm^{-2}; 3D7 and HB3 (not shown); $FCR3^{CSA\text{-}U}$]. As described above, $FCR3^{CSA\text{-}U}$ bound to both ICAM-1 (low) and CSA (>1000 mm^{-2}), but $FCR3^{CSA+}$ bound only to CSA (>8000 iRBC mm^{-2}). No parasite other than $FCR3^{CSA}$ (unselected and CSA selected) bound significantly to CSA. These results confirm that *FCR3.varCSA* transcript levels are not upregulated in parasites selected for enhanced CSA binding, and show that parasites containing full-length *varCSA* transcripts do not necessarily bind to CSA.

Discussion

Our study of *var* gene transcripts in laboratory parasites was designed to resolve an apparent discrepancy between two methods of analysing RNA. Our Northern blot data had suggested that full-length *var* transcripts are present only in ring stages (Kyes *et al.*, 2000), and that these transcripts correlate with the PfEMP1 type expressed (Smith *et al.*, 1998). RT-PCR data had suggested that many *var* types are transcribed in ring stages, and that only the major PfEMP1-encoding type is transcribed in pigmented trophozoites (Chen *et al.*, 1998b; Scherf *et al.*, 1998). Recent investigations, which focus on single-cell RT-PCR with increased sensitivity of *var* gene detection, have suggested that many *var* transcripts can still be detected in pigmented trophozoite stages, and that truly exclusive *var* gene transcription never occurs (Duffy *et al.*, 2002). Although it is powerful for determining which *var* transcripts are present, we felt that RT-PCR is too sensitive for making generalizations about transcription timing. For example, sporozoite stage-specific transcripts can be detected by RT-PCR in asexual stages (Fidock *et al.*, 1994; Chen *et al.*, 1998b; Scherf *et al.*, 1998), suggesting that either stage specificity is not controlled at the transcription level or RT-PCR is too sensitive to distinguish a low background level from full specific activation. Critically, our own RT-PCR experiments on the naturally occurring *varCSA* ‘knock-out’, 3D7, showed that the major *var* transcript detected in pigmented trophozoites is the *varCSA* pseudogene (Taylor *et al.*, 2000). Therefore, until we understand more about how to interpret RT-PCR data, Northern blot analysis paired with phenotype data is essential for unravelling transcriptional control processes. We have concentrated here on verifying our own Northern blot results.

We focused on two possible alternative interpretations of our previously published data: that the RNA sample amounts for pigmented trophozoites were insufficient to detect a low relative level of *var* transcripts, or that the probe was not detecting all *var* transcripts. We

began with testing the timing of steady-state full-length *var* transcripts within asexual stages, in phenotypically unselected A4 parasites. Time course Northern blots comparing either relative transcript levels (in equivalent amounts of total RNA) or absolute levels (in RNA from equivalent numbers of cells) were similar for the majority of *var* transcript types. However, we did find a bias in our 'generic' *var* probe. The use of a new type of *var* probe, *varCSA* exon 2, led to the observation of more *var* transcript types and two different steady-state transcript patterns. One pattern, identical to our previous findings, is exhibited by multiple *var* transcripts of varied sizes, detected only in rings. The other pattern is represented by a unique 15 kb transcript, detected either in all stages or predominantly in pigmented trophozoites. We confirmed, by hybridization with gene-specific probes at high stringency, that the unique 15 kb *var* transcript is the highly conserved *varCSA* gene. Thus, in unselected A4 parasites, there are two steady-state *var* transcript patterns, and the *varCSA* gene pattern is different from that for many other *var* genes.

As this result could be explained by some unique feature of the A4 parasite, and as the *varCSA* gene is highly conserved between isolates (Rowe *et al.*, 2002; Salanti *et al.*, 2002), we investigated whether the *varCSA* transcripts are present in both ring and pigmented trophozoite stages in other genetically related and unrelated laboratory parasites. We compared equal amounts of total RNA in full 48 h time courses and showed that the timing of *varCSA* transcript detection in A4 and related FCR3^{CSA} is similar (low levels in early rings, high levels at late rings and in pigmented trophozoites). In the unrelated TM180 parasite, *varCSA* transcripts are present at similar levels in both rings and pigmented trophozoites. Two-point time courses showed that 3D7 does not transcribe detectable levels of a full-length version of the pseudogene, *3D7chr5var*, and that, in HB3, similar levels of *varCSA* are detected in rings and pigmented trophozoites. This demonstrates that the *varCSA* gene could have two slightly different steady-state transcript patterns, depending on the parasite lineage, with transcripts detectable from early ring stage onwards or mainly in pigmented trophozoites.

Most strikingly, the CSA-binding phenotype did not correlate with the presence of full-length *varCSA* transcripts. Only the FCR3^{CSA-U} and FCR3^{CSA+} infected red blood cells bound to CSA. In these two parasite lines, a sixfold increase in CSA binding did not correlate with any change in the level of the *varCSA* transcript. Additionally, the *varCSA* transcripts were detected in A4, TM180 and HB3, which did not bind to CSA. Therefore, all parasites tested with an intact *varCSA* gene contain full-length *varCSA* transcripts at some point in the 48 h life cycle, regardless of binding phenotype.

One *var* transcript appears to correlate well with CSA binding in FCR3^{CSA-U} and FCR3^{CSA+}. Our starting culture of FCR3^{CSA-U} bound to both ICAM-1 and CSA and contained at least three *var* transcripts in ring stages, of 9.5 kb, 14 kb and 15 kb. After CSA selection of FCR3^{CSA-U}, the enrichment of the 14 kb *var* transcript correlates with an increase in CSA binding and a decrease in ICAM-1 binding. Combined with our data that all parasites can contain the 15 kb *varCSA* transcripts regardless of CSA-binding phenotype, this suggests that the *var* transcript unique to FCR3^{CSA+} is a 14 kb band. We are currently characterizing this transcript. In retrospect, our findings reconcile data from Duffy *et al.* (2002), which suggested the presence of *varCSA* transcripts in both a CSA-selected IT/FCR3 lineage parasite and its non-CSA-binding parent, and indicated a novel *var* transcript type in the CSA-selected parasites (Duffy *et al.*, 2002). The novel *var* transcript agrees in relative size (and stage specificity) with the '14 kb' band that we identified in FCR3.CSA CSA-selected parasites, possibly representing the same *var* gene.

The different pattern of steady-state transcripts for the 15 kb *varCSA* gene suggests that it is unique in its regulation compared with the other *var* genes detected by the complex *var* exon

2 probe, and is in a class of its own for studying rules of *var* gene transcriptional regulation and switching. This is supported by the difference in its upstream regulatory sequence (Vazquez-Macias *et al.*, 2002) compared with other *var* genes (Voss *et al.*, 2000).

Preliminary Northern blot data (not shown) suggest that the full-length conserved *varCSA* gene is transcribed in ring-stage field isolates from children, and RT-PCR detects these transcripts in non-pregnant donors (Fried and Duffy, 2002). *VarCSA* transcripts are therefore not strictly a characteristic of isolates from pregnant women. This does not rule out the possibility that the *varCSA*-encoded PfEMP1 is directly involved in CSA binding, but it does strongly suggest that the *varCSA*-type gene will be detected by RT-PCR in many isolates, whatever the stage and phenotype. In theory, the ring stages will have a higher relative level of the relevant PfEMP1-encoding *var* transcripts. In practice, pigmented trophozoites yield more RNA, so both stages should be valid for investigating repertoires of *var* transcription by RT-PCR. However, our data have important implications for the interpretation of field study data. The most conservative view cautions against correlating the presence of *varCSA*-type transcripts with CSA binding.

Experimental procedures

Laboratory parasites, phenotypes and culturing

Most laboratory parasites were from local cryopreserved stocks. All parasite genotypes were confirmed by PCR. The A4 parasites are genetically identical to FCR3 (Robson *et al.*, 1992). A4 was originally derived from ITO4 selected for binding to ICAM-1 (Roberts *et al.*, 1992). The A4 parasites used in these experiments termed $A4^{ICAM}$ (unselected) or $A4^{ICAM-U}$ had not been selected for any binding phenotype for over 20 cycles in culture. $A4^{ICAM-1C1}$ was chosen as a control for high ICAM-1 binding; it had been derived from a high ICAM-1 binding culture of A4 by limiting-dilution cloning. The parasite $FCR3^{CSA}$ was obtained from the Malaria Research and Reference Reagent Resource Center (MR4; depositor A. Scherf) as a positive control for a CSA-binding parasite, with *FCR3.varCSA* transcription. This frozen parasite stock was thawed, cultured for several weeks, then cryopreserved for storage. After thawing again, it was cultured for three cycles and analysed for RNA and binding phenotype. These parasites, after a total of two thaws and 22 cycles in culture, bound not only to CSA but also to ICAM-1. At 28 cycles (from the MR4 source), the parasites were panned once on CSA, cultured for seven cycles and analysed again for RNA and binding phenotype (a total of 35 cycles in culture with one CSA selection; referred to as $FCR3^{CSA+}$). A parallel unselected culture was compared (at 35 cycles; $FCR3^{CSA-U}$). Panning on CSA bound to plastic (Sigma C9819, 1 mg ml^{-1} in phosphate-buffered saline; 1× PBS: 0.01 M Na-phosphate, 0.0027 M KCl, 0.138 M NaCl, pH 7.4), was performed as described by Roberts *et al.* (1992). TM180, HB3 and 3D7 are unrelated, are not related to IT/FCR3 and had all been grown for at least 10 cycles with no particular phenotype selection.

Parasites were cultured and sorbitol synchronized with standard techniques (Trager and Jensen, 1976; Lambros and Vanderberg, 1979). All cultures were mycoplasma negative (ATCC mycoplasma PCR test kit, according to instructions).

RNA preparation and Northern blots

Parasites were sorbitol synchronized then cultured for at least 2 h before harvesting the first ring-stage RNA samples. Cultures were at 3–10% parasitaemia, and 200–500 μl of packed infected red blood cells were processed for each RNA sample. Cells were spun directly from warm culture medium, the supernatant removed, and the appropriate volume of TRIzol (Invitrogen) was added. Samples were processed as described by Kyes *et al.* (2000).

For the $A4^{ICAM-U}$ time course, samples were taken every 4 h, from the same culture at 9% parasitaemia. The samples were resuspended in equivalent volumes of formamide, and roughly 2×10^7 cell equivalents of total RNA were loaded per lane on the agarose gels in Fig. 1A–D.

For the comparison of ring (16 h after invasion) and pigmented trophozoite (30–36 h after invasion) RNA from different parasite lineages, samples were first electrophoresed on a small 1% agarose gel [containing 1× TBE (0.089 M Tris, 0.089 M boric acid, 2 mM EDTA) and 5 mM guanidine thiocyanate (Goda and Minton, 1995)] to compare relative RNA content, then on 0.8% agarose–1× TBE–5 mM GSCN gels for blotting. Aliquots of 3–6 μg of total RNA were loaded per lane and transferred to Hybond-N^+ (Amersham).

PCR and probes

Standard PCR conditions for amplification from *P. falciparum* genomic DNA were used to produce probe fragments. Primers were varC as reported by Rubio *et al.* (1996), *varCSA* exon 2 (spanning amino acids 3313–3536, referring to map in Buffet *et al.*, 1999) and *varCSA* individual DBL fragments [DBL4, DBL5 and DBL7, as reported by Buffet *et al.* (1999)]. Perkin-Elmer *Amplitaq* DNA polymerase was used with manufacturer's buffer, 2 mM $MgCl_2$. Reactions were cycled: 95°C for 3 min, followed by 30 cycles of 94°C for 30 s, 50°C for 30 s, 65°C for 1 min. Template for all amplification was A4 genomic DNA. For the varC primers, this results in a mix of *var* exon 2 fragments. All double-stranded DNA fragments were purified on Qiagen PCR columns, according to the manufacturer's instructions. The *R29var1* exon 2 plasmid was derived by first cloning a larger PCR fragment amplified from A4 genomic DNA, then subcloning a *Dra*I fragment. Its identity to *R29var1* exon 2 was confirmed by hybridization to Northern blots containing RNA from *R29var1*-transcribing parasites and by restriction mapping, and the sequence (GenBank accession no. AJ535777) matches the partial exon 2 sequence (reported previously, accession no. Y13403). The complex *var* exon 2 probe was a mix of varC and plasmid *R29var1* exon 2. Aliquots of 20–40 ng of DNA were α-^{32}P-dATP labelled (Megaprime; Amersham, according to the manufacturer's instructions).

Hybridizations

Prehybridization (>1 h) and hybridizations (overnight) were performed in 7% SDS–0.5 M $NaPO_4$–2mM EDTA, pH 7.2 (Church and Gilbert, 1984) Low-stringency hybridizations for varC and exon 2 probes were at 50°C; high stringency for *varCSA* DBL probes was at 60°C. Washes were at 5°C more than the hybridization temperature, in 0.5× SSC 0.1% SDS, except where noted otherwise. Exposures to autoradiography film ranged from 1 to 7 days. Band sizes given are approximate and are only indicated to identify bands according to relative size. Absolute size is difficult to determine, as most *var* transcripts are well above the range of the molecular size markers. GELDOC software variously puts the *varCSA* transcript size at 14.3–15.2 kb, depending on the gel.

Densitometry was performed using Bio-Rad GELDOC software.

Binding assay

All laboratory parasites were cryopreserved, thawed, then analysed for binding phenotype according to methods described by Roberts *et al.* (1992). CSA (in 1× PBS, 10 μg ml^{-1} and 100 μg ml^{-1}; Sigma C8529), ICAM-1-FC (40 μg ml^{-1}; Berendt *et al.*, 1992) and 1% bovine serum albumin (BSA) were placed, 3 μl per spot on a Petri dish, incubated at 4°C overnight, then blocked with 1% BSA in 1× PBS for 1 h at 37°C. Parasites were adjusted to 5–6% parasitaemia, resuspended in binding medium (pH 7), then incubated for 1 h on the Petri dishes (with gentle mixing every 10 min). Non-adherent cells were removed by gentle

washing with 1× PBS; plates were fixed in 2% glutaraldehyde, stained with Giemsa, then air dried. Parasite binding levels were calculated as numbers of infected red blood cells mm^{-2}, with duplicate plates in each experiment and triplicate spots per plate. BSA binding levels were subtracted as background for each spot. RNA samples were collected at both ring (16–20 h before assay) and pigmented trophozoite stages (at time of binding assay). Binding assays were repeated several times in subsequent cycles, and the data shown are representative.

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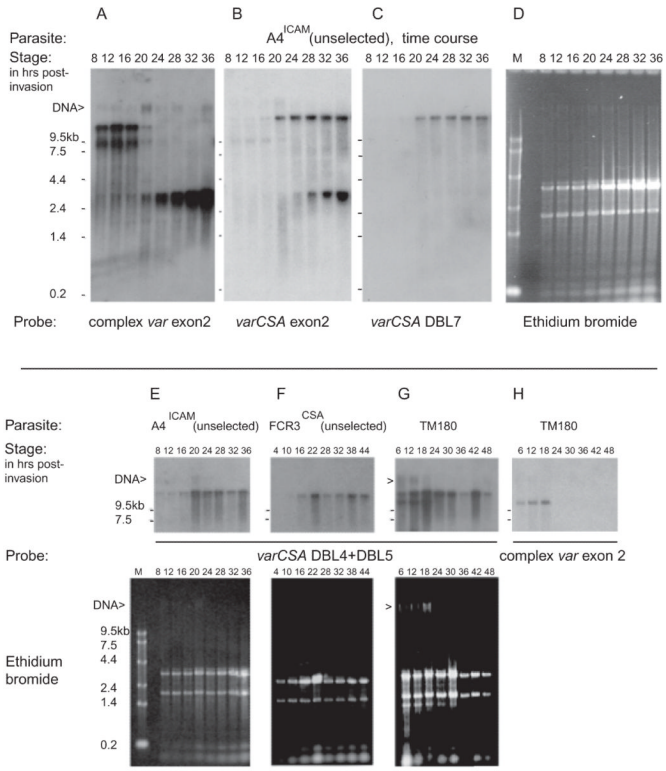

Fig. 1.

Two patterns of *var* transcription. RNA time courses through the 48 h life cycle for various parasites. Stage indicated in hours after invasion. 'DNA>' indicates shadow of cross-hybridization with genomic DNA in the RNA preparation, sometimes visible in the ethidium bromide gel picture.

A–D. Absolute *var* transcript levels during asexual life cycle in $A4^{ICAM-U}$ parasite. Total RNA was compared on duplicate Northern blots, with $\approx 2 \times 10^7$ infected red blood cell equivalents per lane.

A–C. Probes indicated below each blot.

D. Ethidium bromide-stained gel picture shown for comparison of total RNA amounts. Lane M, Invitrogen 0.24–9.5 kb RNA marker.

E–H. Relative *var* transcript levels in $A4^{ICAM-U}$, $FCR3^{CSA-U}$ and TM180 parasites. Total RNA compared with equal amounts per lane (3–5 μg). Ethidium bromide-stained gel pictures below each blot show relative loading levels.

E. $A4^{ICAM-U}$.

F. $FCR3^{CSA-U}$.

G and H. TM180 (same blot).

E, F and G hybridized with *varCSA* DBL4 + DBL5; TM180 blot washed at lower stringency (60°C) than A4 and FCR3 blots (65°C).

H. Hybridized at low stringency with complex *var* exon 2 probe.

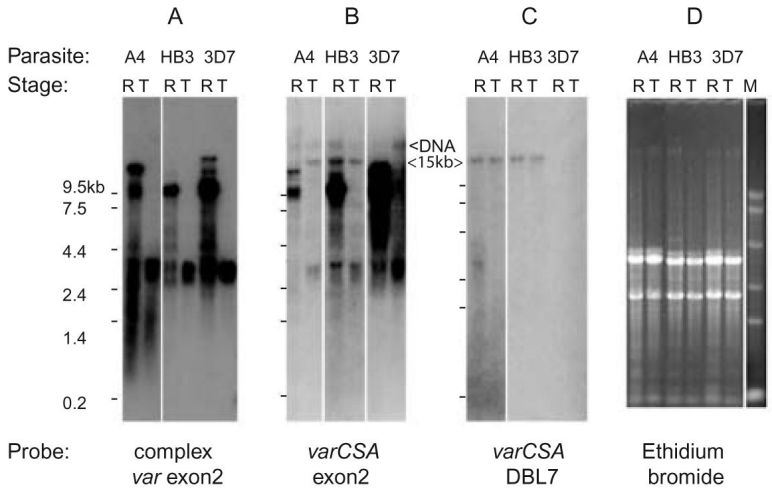

Fig. 2.

Relative *var* transcript levels in genetically unrelated parasites. Duplicate Northern blots with $A4^{ICAM-U}$, HB3 and 3D7 parasite total RNA from mid- to late-ring (R) and pigmented trophozoite (T) stages, 3–5 μg per lane, hybridized as indicated.

A–C. Probes indicated below each blot. D. Ethidium bromide-stained gel picture shown for comparison of total RNA amounts. 'DNA>' indicates genomic DNA in the RNA preparation.

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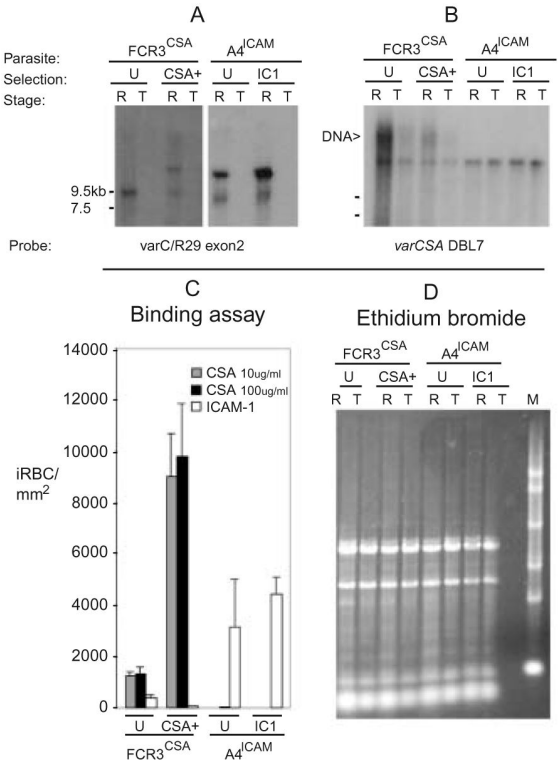

Fig. 3.

Detection of constitutive *varCSA* transcript in parasites that do not bind to CSA. Parasites of the same genotype, with different binding phenotypes, were sampled for RNA at ring stage (≈ 16 h after invasion). Twenty hours later, pigmented trophozoite-stage parasites were harvested for both RNA and binding assays. Binding data shown in (C) were from the same cultures sampled for RNA (except for $A4^{ICAM\text{-}IC1}$, the binding assay was performed two cycles after the RNA was collected).

A, B and D. Northern blot with $FCR3^{CSA\text{-}U}$ (U, unselected), $FCR3^{CSA+}$ (CSA+, CSA selected), $A4^{ICAM\text{-}U}$ (U, unselected) and $A4^{ICAM\text{-}IC1}$ (IC1, ICAM-1 binding), 3–5 μg of total RNA per lane. Ring (R) and pigmented trophozoite stages (T). Probes indicated below each blot.

D. Ethidium bromide pattern shows relative total RNA levels. ‘DNA>’ indicates cross-hybridization with genomic DNA.

C. Binding assay. Binding is presented as the number of infected red blood cells mm^{-2} (iRBC mm^{-2}), with standard deviation. 10 μg ml^{-1} CSA (grey bars), 100 μg ml^{-1} CSA (black bars) and 40 μg ml^{-1} ICAM-1-FC (open bars).