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# Transcribed *var* Genes Associated with Placental Malaria in Malawian Women†

Michael F. Duffy,[1]‡* Aphrodite Caragounis,[1]‡ Rintis Noviyanti,[2] Helen M. Kyriacou,[3] Ee Ken Choong,[1] Katja Boysen,[1] Julie Healer,[1]§ J. Alexandra Rowe,[3] Malcolm E. Molyneux,[4,5] Graham V. Brown,[1] and Stephen J. Rogerson[1]

*Department of Medicine (RMH), University of Melbourne, Melbourne, Australia[1]; The Eijkman Institute for Molecular Biology, Eijkman Building, Jl. Diponegoro 69, Jakarta, Indonesia 10430[2]; Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, King's Buildings, West Mains Rd., Edinburgh, United Kingdom[3]; Malawi-Liverpool-Wellcome Trust Clinical Research Programme, College of Medicine, University of Malawi, Blantyre, Malawi[4]; and School of Tropical Medicine, University of Liverpool, Liverpool, United Kingdom[5]*

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**Determining the diversity of PfEMP1 sequences expressed by *Plasmodium falciparum*-infected erythrocytes isolated from placentas is important for attempts to develop a pregnancy-specific malaria vaccine. The DBLγ and *var2csa* DBL3x domains of PfEMP1 molecules are believed to mediate placental sequestration of infected erythrocytes, so the sequences encoding these domains were amplified from the cDNAs of placental parasites by using degenerate oligonucleotides. The levels of specific *var* cDNAs were then determined by quantitative reverse transcription-PCR. Homologues of *var2csa* DBL3x were the predominant sequences amplified from the cDNAs of most placental but not most children's parasites. There was 56% identity between all placental *var2csa* sequences. Many different DBLγ domains were amplified from the cDNAs of placental and children's isolates. *var2csa* transcripts were the most abundant *var* transcripts of those tested in 11 of 12 placental isolates and 1 of 6 children's isolates. Gravidity did not affect the levels of *var2csa* transcripts. We concluded that placental malaria is frequently associated with transcription of *var2csa* but that other *var* genes are also expressed, and parasites expressing high levels of *var2csa* are not restricted to pregnant women. The diversity of *var2csa* sequences may be important for understanding immunity and for the development of vaccines for malaria during pregnancy.**

Malaria is more common in pregnant than nonpregnant women and predisposes those infected to maternal and infant death (37). The placenta provides a unique site for infected erythrocytes (IE) to be sequestered through adhesion to receptors, including chondroitin sulfate A (CSA) and hyaluronic acid (3, 14). IE from nonpregnant individuals rarely adhere to CSA and hyaluronic acid (3). The restricted adhesion phenotype of parasites isolated from placentas is associated with the expression of a restricted repertoire of parasite variant surface antigens (VSAs). Consequently, with increasing parity, women acquire antibodies that bind to parasites isolated from their own and other placentas (1, 16). These antibodies are associated with protection (11, 16, 36), and some are capable of blocking IE adhesion to CSA (2, 16, 39). It has therefore been postulated that parasite sequestration in the placenta is caused by a conserved, placenta-specific subset of VSAs.

Adhesion of IE is mediated predominantly by the diverse *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) molecules encoded by the *var* multigene family. Each parasite possesses approximately 60 *var* genes (20), and allogeneic parasites possess different repertoires of *var* genes. Previous studies linked adhesion to CSA to several *var* genes, including *var-CS2* (29) and *FCR3.varCSA* (6). In both cases, adhesion was attributed to the DBLγ domain of PfEMP1. However, subsequent studies have not corroborated these findings and suggest that transcription of at least *FCR3.varCSA* is not associated with adhesion to CSA (9, 10, 25, 35, 40, 41). A more promising candidate CSA binding parasite ligand is the VAR2CSA PfEMP1 identified by Salanti et al. (36). The most abundant *var* transcript in multiple allogeneic CSA-adherent parasites is *var2csa* (9, 12, 35), these parasites are antigenically cross-reactive (12), and evidence of parity-dependent antibody binding to defined, CSA-adherent laboratory isolates is currently restricted to parasites that express *var2csa* (2, 35). However, the identity of the conserved, CSA-binding parasite antigen remains unresolved because parity-dependent antibodies bind multiple recombinant proteins derived from both domains of VAR2CSA (33) and the DBLγ domain of at least one other PfEMP1 (5, 7). Furthermore, DBLγ domains present in multiple PfEMP1 molecules and multiple domains of VAR2CSA all bind to CSA (6, 18, 30).

Previous nonquantitative studies of the *var* genes expressed by placental isolates have either used degenerate primers designed to amplify multiple DBLγ domains or specific primers to amplify DBL domains from some of the three *var* genes identified as candidate CSA adhesion ligands (15, 22, 23, 32). The amplification of multiple sequences and lack of quantita-

* Corresponding author. Mailing address: Department of Medicine (RMH), University of Melbourne, Post Office, Royal Melbourne Hospital, Victoria 3050, Australia. Phone: 61 3 8344 3267. Fax: 61 3 9347 1863. E-mail: mduffy@unimelb.edu.au.

† Supplemental material for this article may be found at http://iai.asm.org/.

§ Present address: The Walter and Eliza Hall Institute of Medical Research, 19 Royal Parade, Parkville, Victoria, Australia 3050.

‡ M.F.D. and A.C. contributed equally to this work.

tive data prevented the detection of a clear association between a specific *var* gene and placental sequestration. These studies also did not detect *var2csa* because it lacks the DBLα and DBLγ domains for which the degenerate primers were designed. Quantitative analyses of *var* transcription in placental isolates have been restricted to comparisons of *var2csa* and *FCR3.varCSA* (35, 40). These studies were suggestive of a major role for *var2csa* in malaria during pregnancy and excluded a similar role for *FCR3.varCSA* but did not address the potential roles of other *var* genes possessing DBLγ domains that may also have been transcribed at high levels in placental isolates. We used degenerate primers capable of amplifying both DBLγ domains and *var2csa* DBL3x domains to amplify a range of transcribed *var* genes from parasites isolated from placentas and from the peripheral blood of children. We then used quantitative reverse transcription (Q-RT-PCR) on cDNAs from the parasite isolates to determine the levels of multiple *var* gene transcripts, including DBLγ sequences, that had been amplified from the cognate cDNAs.

## MATERIALS AND METHODS

**Parasites.** Informed consent was obtained from all patients or (for children) their parents or guardians for participation in the study. The study was approved by the College of Medicine Research and Ethics Committee, University of Malawi, and the Royal Melbourne Hospital Clinical Research Ethics Committee. *P. falciparum*-infected blood was obtained from placental biopsies from 19 women, as previously described (3), and from peripheral veins of six children admitted with severe malaria at the Queen Elizabeth Central Hospital in Blantyre, Malawi (31). Placental and peripheral blood samples were washed, and the pellets were solubilized at a 1-in-10 dilution in Trizol (Invitrogen, Carlsbad, CA) and stored frozen in liquid nitrogen. The Rplc series of placental isolates was collected from 1997 to 1999 (32), while the Mplc placental samples and the Mch children's samples were collected from 1999 to 2001 (31).

**DNA and total RNA preparation.** Genomic DNA was isolated from 100 μl of packed erythrocytes by using a QIAmp DNA mini blood kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. For RT-PCR, RNAs were extracted from infected erythrocyte pellets solubilized in Trizol as described previously (9). RNAs were treated for 2 h at 37°C with DNase I (4 U; Ambion, Austin, TX) in the presence of total RNA carrier (Sigma, Sydney, Australia) and with 40 U of RNasin RNase inhibitor (Promega, Madison, WI).

**PCR and RT-PCR assays.** Reverse transcription was performed as previously described (9). Reverse transcriptase negative controls were used for each sample. DBLγ and *var2csa* DBL3x sequences of approximately 600 bp were amplified from cDNAs and genomic DNAs (gDNAs) from children's and Mplc series placental samples by PCRs using the degenerate oligonucleotides D3F (CCTC CWAGRAGAMAAAAATTAT) and D3R1 (RCAAAAITSITCICKCCATTC) (I stands for inosine). PCR was performed under the following conditions: 94°C for 5 min, 40 cycles of 94°C for 30 s, 48°C for 30 s, and 65°C for 30 s, and a final extension at 65°C for 6 min. PCR mixtures included 25 pmol of each primer, a 500 nM concentration of each deoxynucleoside triphosphate, and 2.5 mM $MgCl_2$. The Rplc series of samples was analyzed some time after the Mplc samples, allowing an improved degenerate reverse primer (D3R1.2 [ACAAWANTSNT CDBMCCATTC]) to be designed for amplification of the Rplc samples. D3R1.2 differed slightly in degeneracy from D3R1 but bound the same sequence.

To obtain additional DBLγ sequences from some samples, it was necessary to use a seminested PCR that could only amplify DBLγ sequences. The seminested PCR was performed in 50 μl under the conditions described above, and the mixture contained 0.5 μl of the first-round PCR product as the template and the primers D3F and D3R2 (CCATCKIARAAATTGIGGTYT). The use of a seminested PCR increased the bias of amplification of particular sequences, but this was controlled for by subsequent quantitative analyses.

cDNAs from the children's isolates Mch1478 and Mch 1488 and from the placental isolates from which *var2csa* cDNA could not be amplified using the degenerate primers were subjected to PCR using a *var2csa* DBL3x-specific forward primer (ACGATAAAGGTACAGCAATTATAT) and the same reverse primer as that used for Q-RT-PCR (TGGTACCCAAAATCATATTCTTATA ATCA).

All PCR products were cloned and sequenced using standard methods.

**Q-RT-PCR.** Absolute quantitation of *var* gene cDNAs by Q-RT-PCR was performed as previously described, using standard curves of purified plasmids containing *var* gene sequences (9). Briefly, each PCR used 5 μl SYBR green PCR master mix (PE Biosystems) in a 10-μl reaction mix that was amplified using the ABI Prism 7900HT sequence detection system. PCR mixtures were incubated at 95°C for 10 min and then subjected to 40 cycles of 95°C for 15 s and 60°C for 1 min. A final incubation of 95°C for 2 min was followed by a dissociation step at 60°C for 2 min, with a 2% ramp rate to 95°C for 2 min. The specificity of each primer pair was determined by dissociation curve analysis per the manufacturer's instructions. The primers used to amplify *var2csa* DBL3x (forward, TGTCATG CTGTTCAAAGAAGTTTTATT; and reverse, TGGTACCCAAAATCATATT CTTATAATCA) were designed using an alignment of the 3D7 and ItG *var2csa* sequences and the 24 *var2csa* sequences amplified in this study. Nucleotide 16 of the DBL3x reverse primer had a C-for-T mismatch with Mplc22.5, and nucleotide 2 had a T-for-G mismatch with Rplc132c, Rplc132l, Rplc143d, and Rplc143e. However, *var2csa* DBL3x sequences with 100% identity to the *var2csa* DBL3x forward and reverse primers were also amplified from the same isolates. Nucleotide 11 of the DBL3x forward primer had a C-for-T mismatch with the only *var2csa* sequence amplified from Mplc78. Consequently, for quantitation of *var2csa* in Mplc78 cDNA, the DBL3x forward primer was replaced with TGTC ATGCTGCTCAAAGAAGTTTTATT, and the cloned Mplc78 *var2csa* sequence was used to construct the standard curve. The primers used to amplify *var-CS2* (forward, AGGAGATCAAGCAACCAC; and reverse, TTATTATCATCAAT GTGTGTATCTCCTTTT) were identical to the *var-CS2* sequence and the two sequences amplified in this study from Mplc78. The primers used to amplify *FCR3var.CSA* had the following sequences: TTGGAGGAATTTGCCAAACG (forward) and CCATTCGGTTAGCCATCGTAAA (reverse). The quantities in each cDNA of the skeleton binding protein 1 gene (SBP) (PFE_0065w) (forward, TTAGCCGACGAACCAACACA; and reverse, TTCGGTTGTCTCTGGTAC TGCA) and a hypothetical protein gene (PF11_0505) (forward, TTTCAAGGG TCCAAGTTATGATGA; and reverse, AGGGCTTTGCCAAAAACTTTC) were determined by Q-RT-PCR, using standard curves of diluted 3D7 isolate gDNA. SBP and PF11_0505 levels were used to normalize the quantitative *var* cDNA data. Other primers used are shown in Table 1.

**Sequence analysis.** Sequence analysis was performed using BioEdit (21). Clustal W multiple sequence alignments with 100 bootstrap replicates were used to create protein distance matrices and phylogenetic trees by the neighbor-joining method (13) available at http://evolution.genetics.washington.edu/phylip.html and by using TreeView (28), available at http://taxonomy.zoology.gla.ac.uk/rod/treeview.html.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for all of the sequences reported in this study are AY461586 to AY461594, AY464699, AY464700, AY466363 to AY466367, AY466369 to AY466372 AY488868 to AY488880, AY534318, AY534319, DQ286586 to DQ286636, and DQ306266 to DQ306307.

## RESULTS

The ability of the degenerate oligonucleotides D3F and D3R1 (Fig. 1a) to amplify both DBLγ and *var2csa* DBL3x sequences was tested by cloning products amplified from 3D7 genomic DNA. Forty clones were sequenced, yielding 9 of the 14 published 3D7 DBLγ sequences (26) and the *var2csa* DBL3x sequence. After 40 cycles of amplification, the most frequently amplified sequence (PFD0005w) was detected at 1.5 times the expected frequency using a binomial distribution, as previously described (38) (Fig. 1b). Using the analysis of Taylor et al. (39), these degenerate primers showed a low bias towards amplification of the most frequently detected sequence of $<2\%$ (since $1.02^{40}$ is $>1.5$), which is consistent with those of other "universal" DBL domain degenerate primers (8, 24, 38). Nevertheless, the bias may prevent the amplification of some DBLγ sequences. The *var2csa* DBL3x sequence was amplified at a moderate frequency (Fig. 1b).

The oligonucleotides D3F and D3R1/D3R1.2 were then used to amplify DBLγ and *var2csa* DBL3x sequences from the cDNAs of 10 placental isolates and 4 isolates from the peripheral blood of children and from the gDNAs of 4 placental

TABLE 1. Data for placental and children's isolates examined in this study[a]

| Group | Sample | Reverse primer[b] | No. of sequences amplified from: | | | | Sample used for Q-RT-PCR | Endogenous DBLγ control for Q-RT-PCR | Primer sequence for endogenous DBLγ control | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | cDNA | | gDNA | | | | | |
| | | | *var2csa* DBL3x[c] | DBLγ[d] | *var2csa* DBL3x | DBLγ | | | Forward | Reverse |
| A | Rplc132 | D3R1.2 | 6 | 2 | ND | ND | No | ND | | |
| A | Rplc134 | D3R1.2 | 2 | 6 | ND | ND | No | ND | | |
| A | Rplc136 | D3R1.2 | 1 | 4 | ND | ND | No | ND | | |
| A | Rplc143 | D3R1.2 | 2 | 5 | ND | ND | No | ND | | |
| A | Rplc154 | D3R1.2 | 2 | 5 | ND | ND | No | ND | | |
| A | Rplc294 | D3R1.2 | 1 | 7 | ND | ND | No | ND | | |
| A, D | Mch1485 | D3R1 | 0 | 2 | ND | ND | Yes | Mch1485-1 | TTATGAACAACACAAATAAAAACACCTTT | CATCACTGAATTTCACAGTGCCA |
| A, D | Mch1481 | D3R1 | 0 | 5 | ND | ND | Yes | Mch1481-4 | AAAAAGCTGGAGGAAACGACAG | CCGTGTTGTAGTTGTAAGTGGATTTG |
| A, D | Mch1482 | D3R1 | 0 | 4 | ND | ND | Yes | Mch1482-2 | ATAATAACAGACGATAATTTGAAAGATGCT | TGCTGCTGCAGTTCTAATAAAAGC |
| A, C, D | Mch1476 | D3R1 | 0 | 3 | 0 | 2 | Yes | Mch1476-1 | ATGAGAAAGAAATAGAGAAAATGCGAA | AGTTGTAGGTCATGTTCAGTTGTGTTC |
| A, C, D | Mplc226 | D3R1 | 2 | 3 | 0 | 6 | Yes | Mplc226-5 | GTATCACGAGAAGATTGGTGGAAA | CCCTTCCCATATTTGAGGACAA |
| A, C, D | Mplc154 | D3R1 | 2 | 1 | 0 | 3 | Yes | Mplc154-1 | GTCTTTCCGCAAACTGCGA | CATTCACTGGTATTCCCAGTGGT |
| A, C, D | Mplc22 | D3R1 | 5 | 0 | 0 | 4 | Yes | No control | | |
| A, C, D | Mplc21 | D3R1 | 2 | 0 | 0 | 2 | Yes | No control | | |
| B, C, D | Mplc78 | D3R2 | 0 (1) | 2 | 0 | 6 | Yes | *var*-CS2 | | |
| B, C, D | Mplc178 | D3R2 | 0 (1) | 1 | 0 | 3 | Yes | Mplc178-1 | TGGGATGAATATGCTCCAGCT | TGATAGCCCACATAACATTCCTTG |
| B, C, D | Mplc132 | D3R2 | 0 (0) | 1 | 0 | 3 | Yes | Mplc132-1 | AAGGGTCCCAAGAAAGAACTAGATAA | TCCCCACCAAGCTACACGTT |
| B, C, D | Mch1488 | D3R2 | 0 (1) | 3 | 0 | 7 | Yes | Mch1488-3 | ATAATAACAGACGATAATTTGAAAGATGCT | TGCTGCTGCAGTTCTAATAAAAGC |
| B, C, D | Mch1478 | D3R2 | 0 (1) | 3 | 0 | 3 | Yes | Mch1478-2 | AATAATGGCGGCAAATCAGG | TTTCCACCAGTCTTCGCGTT |
| B, D | Mplc176 | D3R2 | 0 (1) | 2 | ND | ND | Yes | Mplc176-1 | ATTTATTCCATAACAAAAAAGTTTCTGCT | ACGCATTTATTAAATGTGCTGCA |
| B, D | Mplc345 | D3R2 | 0 (1) | 1 | ND | ND | Yes | Mplc345-1 | GATGCAAAGTCCTCGAGAGTAGATG | CAAAGACTACCAATATTCCACCGTT |
| B, D | Mplc366 | D3R2 | 0 (1) | 2 | ND | ND | Yes | Mplc366-2 | GCCACTGAATTTGACGGTGG | TGCACTTACTAACAAGGACACCTACAC |
| B, D | Mplc129 | NP | (1) | | | | Yes | No control | | |
| B, D | Mplc159 | NP | (1) | | | | Yes | No control | | |
| B, D | Mplc328 | NP | (1) | | | | Yes | No control | | |

[a] NP, no PCR product; ND not done. Samples with the prefixes Mplc and Rplc are placental isolates, and samples with the prefix Mch are children's isolates.
[b] The D3R1 degenerate primers were capable of amplifying both the DBLγ and *var2csa* DBL3x domains, while the nested D3R2 primers were only capable of amplifying DBLγ domains.
[c] Number of different sequences with at least 77% identity to 3D7 *var2csa* DBL3x that were amplified from an isolate with the degenerate primers. Numbers in parentheses indicate the numbers of *var2csa* sequences amplified by the specific *var2csa* primers.
[d] Number of different DBLγ sequences that were amplified from the isolate.

isolates and 1 child's isolate (Table 1, sample group A). No products could be amplified from the cDNAs of a further nine placental and two children's isolates (Table 1, sample group B). The PCR products were cloned and sequenced (Table 1, sample group A). A range of sequences were amplified from the cDNAs of most isolates, but there was an obvious pattern in the amplification of *var2csa* DBL3x sequences from the cDNAs of all 10 placental isolates but not from the four children's isolate cDNAs (Table 1, sample group A) or the four placental gDNAs amplified with D3R1 (Table 1, sample group A).

We wished to extend this analysis beyond previous studies by first determining the endogenously transcribed DBLγ *var* sequence(s) in each isolate and then quantitating the level of such a sequence in the cDNA of its cognate isolate to compare with the levels of several *var* sequences that have been implicated in adhesion to CSA in laboratory studies, including *var2csa* DBL3x. To do this, we needed to clone DBLγ sequences from the isolates. Therefore, we used the degenerate reverse primer D3R2, which could amplify only DBLγ sequences (Fig. 1), in a second round of RT-PCR amplification. The second-round RT-PCR was performed on the two children's isolates and nine placental isolates from which no detectable first-round product had been amplified using D3R1 (Table 1, sample group B) and on the placental isolates Mplc21 and Mplc22, from which only *var2csa* sequences had been amplified using D3R1. The second-round primer D3R2 amplified DBLγ sequences from the cDNAs of both children's isolates and six of the nine placental isolates (Table 1, sample group B) but not from the cDNAs of Mplc21 and Mplc22. Combining the results from all degenerate primer PCRs, DBLγ sequences were amplified from the cDNAs of 14 of the 19 placental isolates and all six of the children's isolates (Table 1). There was a wide diversity in the DBLγ sequences amplified from the cDNAs of all samples. Sequences were also amplified by either D3R1 or D3R2 from the gDNAs of a randomly selected group of 10 isolates (Table 1, sample group C) to test whether the sequences amplified from cDNAs simply reflected primer bias. However, the sequences amplified from

a

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                    D3F                                 D3R2                                D3R1
AL010226   dbl3gamma CCACCCAGAAGAGAAAAATTTT...AGACCTCAATTCCTTCGATGG...GAATGGAGTGATGAATTTTGT
var-CS2    dbl2gamma CCTCCTAGAAGACAAAAATTAT...AAACCACAATTTCTCCGTTGG...GAATGGAGTGATGAATTTTGT
A4tresvar  dbl3gamma CCACCAAGAAGACAAAATTTAT...AGACCCCCATTTTTACGATGG...GAATGGGGAGAAGA-TTTTGT
FCR3varCSA dbl3gamma CCCCCTAGAAGACAAAAGTTAT...CGACCCCAGTTTTTACGATGG...GAATGGTACGACCGACTATTGC
It4var4    dbl3x    CCGCCAAGAACACAAAATTTAT...ATGATTCAGTCCGTTTCGTGG...GAATGGGGCGAACAGTTTTGT
                    CCTCCWAGRAGAMAAAAATTAT...ARACCICAATTTYTIMGATGG...GAATGGMGIGAISAITTTTGY
                    ** ******* ****** ****...*    *** *   **   ***...****** **************
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b

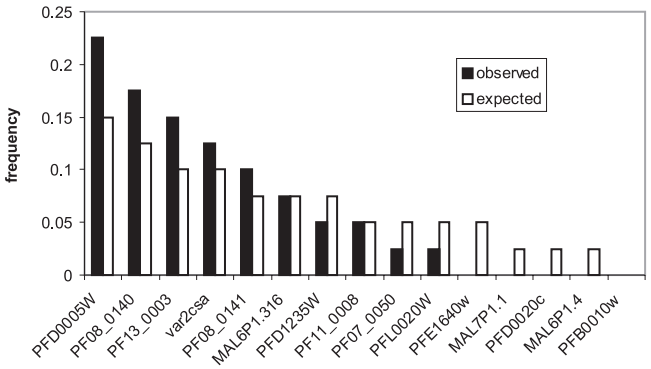


FIG. 1. (a) Alignment of degenerate oligonucleotides D3F, D3R1, and D3R2 with four DBLγ sequences that all bind CSA as recombinant proteins (18) and with the DBL3x domain of the ItG *var2csa* homologue (It4var4). Asterisks under the sequences indicate that the nucleotides at those positions of the oligonucleotides had homology with both DBLγ and DBL3X sequences. W, A or T; R, A or G; M, A or C; Y, C or T; S, G or C; I, inosine. (b) Observed and expected frequencies of detection of *var2csa* and different DBLγ sequences in cloned PCR products amplified from 3D7 genomic DNA using the oligonucleotides D3F and D3R1. Excel BINOMDIST was used to calculate the expected binomial distribution for 40 sequenced clones with 15 DBLγ or DBL3x possible identities if each identity was equally likely (38).

gDNAs were different from those amplified from the cDNAs of the same isolates, suggesting that the sequences amplified from the cDNAs were detected because of their abundance.

An obvious association between placental isolate cDNA and *var2csa* was revealed by a phylogenetic analysis that included all of the amplified sequences and all of the PfEMP1 domains previously implicated in pregnancy-associated malaria (6, 17, 23, 29, 32, 35) (Fig. 2). There was >76% identity between 3D7 *var2csa* and any of the 24 sequences homologous to *var2csa* DBL3x identified in this study. However, only 56% of all 194 predicted amino acids were identical between all of the 22 full-length *var2csa* DBL3x sequences (excluding two sequences that had large deletions) (see Fig. S1 in the supplemental material). Four other DBLγ domains associated with malaria in pregnancy formed small, well-supported clusters: *FCR3var.CSA* DBL5γ had at least 70% identity to four sequences from different placental cDNAs; *FCR3var.CSA* DBL3γ had >96% identity with two sequences from different placental isolate cDNAs and one sequence from a child's isolate cDNA; 3D7chr5var DBL3γ (32), homologous to 720 (23) and $var_{COMMON}$ (42), had >79% identity with three sequences from different placental cDNAs; and *var-CS2* had >97% identity with the only two sequences amplified from a single placental cDNA.

We performed absolute quantitation by Q-RT-PCR to determine quantitative relationships between selected pregnancy-associated *var* sequences within those isolates for which sufficient cDNA was available (Table 1, sample group D). This group comprised all 6 children's isolates and 13 of the placental isolates, including all 9 of the placental samples from which no *var2csa* sequences were amplified with the degenerate primers (Table 1, sample group B). For each cDNA, we quantitated the levels of *var2csa* DBL3x, *var-CS2* DBL2γ, and *FCR3.varCSA* DBL3γ. For all six children's isolates and eight of the placental isolates, we also quantitated the level of an endogenous control DBLγ sequence that had been amplified from the cDNA of the isolate being examined (Table 1 and Fig. 2). This controlled for the possibility that differences between isolates in the levels of transcripts of any of the pregnancy-associated *var* sequences merely reflected differences between isolates in the levels of general *var* gene transcription. The selected pregnancy-associated *var* sequences could not be used to control for this possibility because the presence of these sequences had not actually been established for all isolates. For five placental samples, no endogenous control was used because no sequences could be amplified using the DBLγ degenerate primers. For sample Mplc78, the *var-CS2* DBLγ sequence that was cloned from its cDNA was used as the endogenous control.

Q-RT-PCR revealed that *var2csa* was the most abundant of the transcripts examined for 12 of the 13 placental isolates (sample group D) but that *var-CS2* was a more abundant transcript than *var2csa* in the remaining placental isolate, Mplc78 (Fig. 3). *FCR3var.CSA* was present at a similar level to that of *var2csa* in the cDNA from the placental isolate Mplc328. The endogenous control DBLγ sequence was the most abundant of the transcripts tested in four of the six children's isolate cDNAs, and *var2csa* was the most abundant of the transcripts tested in the other two children's isolate cDNAs (Mch1478 and Mch1488) (Fig. 3). To confirm the presence of *var2csa* sequences in samples analyzed by Q-RT-PCR, we cloned and sequenced *var2csa* PCR products that were amplified with specific primers from the cDNAs of Mch1478, Mch1488, and eight of the nine placental isolates from which *var2csa* could not be amplified using the degenerate primers (Table 1, sample group B). All but one of the cloned *var2csa* sequences were identical to the *var2csa* sequence of the 3D7 isolate within the region that was used as a template for Q-RT-PCR. The *var2csa* sequence cloned from Mplc78 had a single mismatch of C versus T at nucleotide 11 of the *var2csa* DBL3x Q-RT-PCR

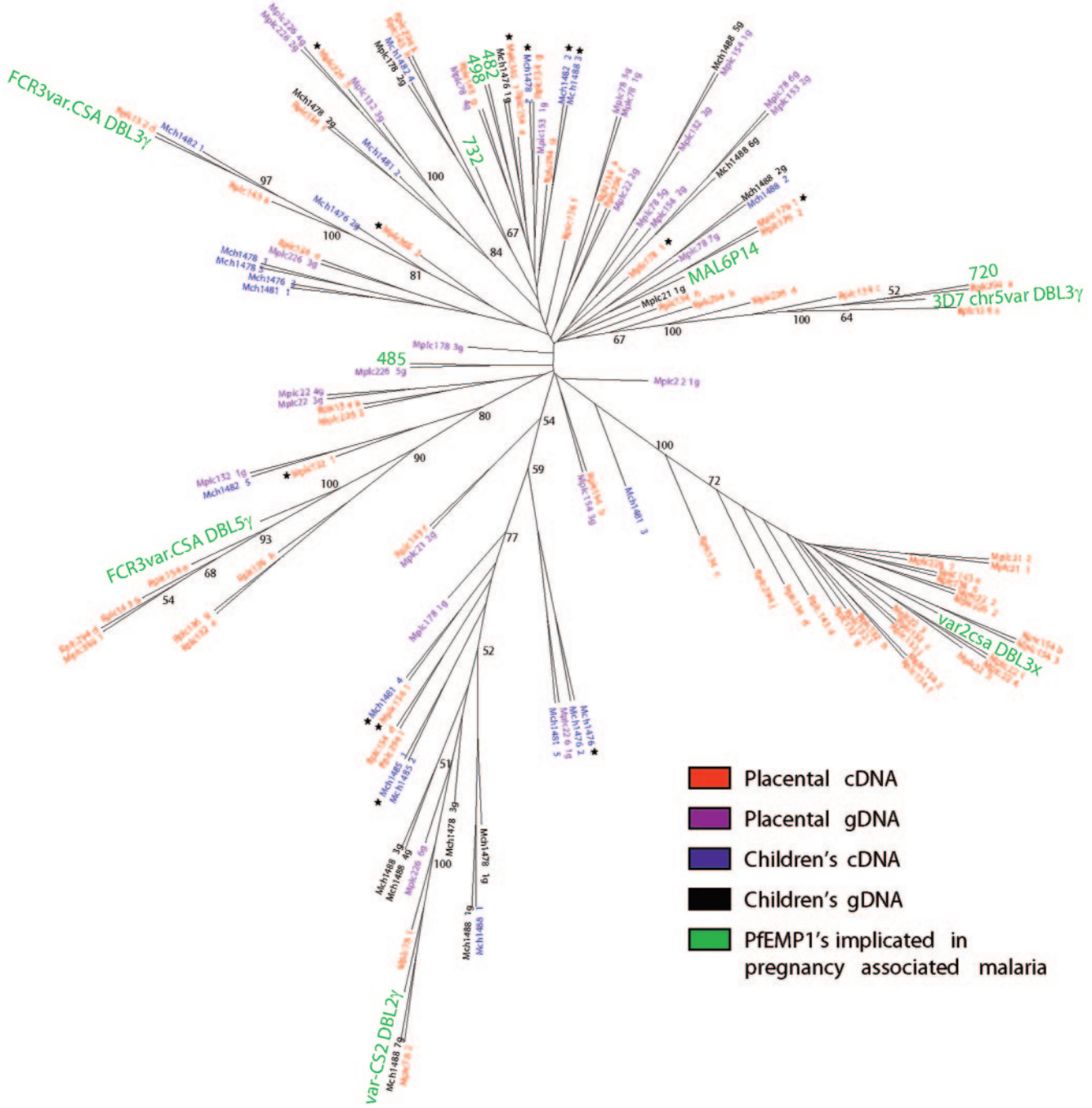


FIG. 2. Phylogenetic tree of sequences amplified from cDNAs and gDNAs of parasites isolated from placentas and from peripheral blood of children. The candidate CSA-binding PfEMP1s encoded by *var2csa* (35), *FCR3.varCSA* (6), *var-CS2* (29), MAL6P14 (17), 3D7chr5var (32), 482, 498, 485, 720, and 732 (7, 23) are included, and those shown in bold are in the clusters described in the text. Bootstrap values of >50 are indicated at tree branches leading to clusters of sequences. The sequences used as endogenous DBLγ controls for Q-RT-PCR are indicated with stars. The sequences were named for the samples from which they were amplified, followed by a space and then a different number for each different sequence from a single Mplc or Mch sample or a different letter for each different sequence from an Rplc sample. The suffix "-g" following an Mplc or Mch sequence indicates that it was amplified from gDNA.

forward primer. Consequently, *var2csa* cDNA was quantitated in the placental isolate Mplc78 by using both a modified *var2csa* DBL3x forward primer that had a cytidine residue at nucleotide 11 and a standard curve constructed from the cloned Mplc78 *var2csa* sequence.

The absolute quantitation data were normalized to compare the cDNA levels of specific *var* sequences between isolates and to indicate the abundances of specific *var* sequences within an isolate. The quantitative data should ideally be normalized to the total level of *var* mRNA to control for fluctuations between isolates in the levels of *var* gene transcripts caused by either differences in the temporal stages of the parasites sampled or

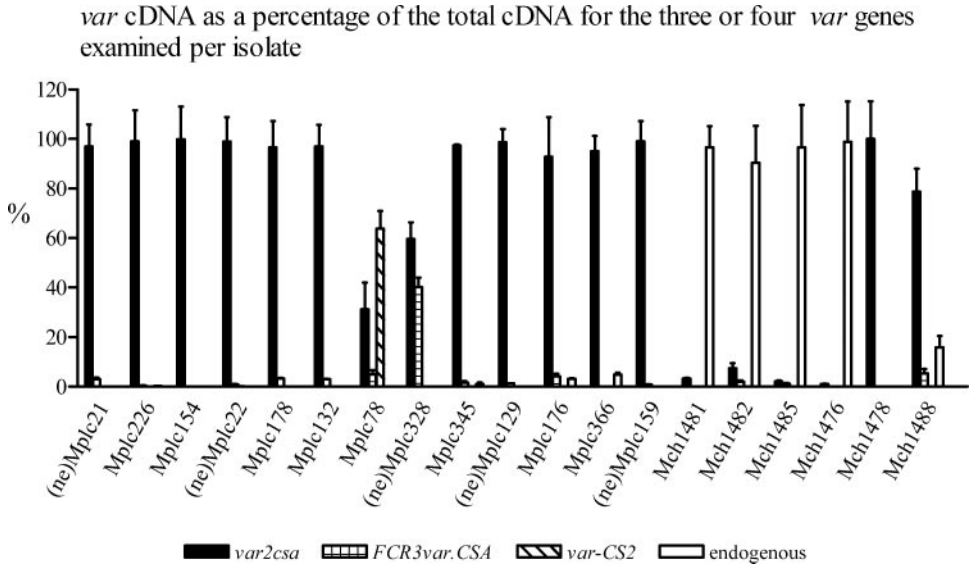


FIG. 3. Percentage that each *var* gene constituted of the total *var* cDNA for the three or four *var* genes examined in each isolate by Q-RT-PCR absolute quantitation. Three *var* genes were quantitated for Mplc78 and for the five placental isolates with the prefix "(ne)-," from which no endogenous control DBLγ sequence could be amplified; four *var* genes were quantitated for the other 13 isolates. Endogenous refers to the endogenous DBLγ sequence that was amplified from the sample being analyzed.

differences in the peak levels of *var* mRNA transcribed by allogeneic isolates. This was an issue in the current study because the peripheral blood samples from children contained predominantly ring-stage parasites at the peak of *var* transcription while the placental isolates were composed mainly of mature trophozoites, in which *var* transcription is reduced. However, the concentration of total, functional *var* cDNA cannot be determined by Q-RT-PCR because the only sequences that are sufficiently conserved between all *var* genes to be used for this purpose are the second-exon sequences, which are also present at high levels in mature parasites as sterile, truncated transcripts. Consequently, to normalize the *var* Q-RT-PCR data, we used two genes, namely, the skeleton binding protein 1 gene (SBP) (PFE_0065w) and a hypothetical protein gene (PF11_0505), because their temporal transcriptional profile closely approximated that of the *var* multigene family in allogeneic parasite strains 3D7, HB3, and Dd2 (4, 27). Therefore, the normalized data presented in Fig. 4b represent the levels of cDNAs for specific *var* genes in equivalent amounts of total *var* cDNA from each isolate, as approximated by two control genes.

We used a time course of CS2 parasites, in which *var2csa* is the dominant *var* transcript (9), to validate our choice of genes for normalization (Fig. 4a). The levels of the normalizing genes were compared to the levels of *var2csa* in the 8-h postinvasion ring-stage CS2 parasites and the 36-h postinvasion mature trophozoite-stage CS2 parasites, using the following ratio: ring-stage *var2csa* cDNA/ring-stage control gene cDNA:mature trophozoite-stage *var2csa* cDNA/mature trophozoite-stage control gene cDNA. This gave ratios of 2.5:1 for SBP and 1.3:1 for PF11_0505. Thus, we assumed that the mature trophozoites in the placental isolates would contain 2.5-fold more *var* transcripts than were calculated by using SBP for normalization and 1.3-fold more *var* transcripts than were calculated by using PF11_0505 for normalization, and we adjusted the normalized data accordingly. The levels of 18S rRNA were also quantitated in the CS2 parasites to show that the similar transcriptional profiles of *var2csa*, PF11_0505, and SBP did not merely reflect the use of decreased parasite cDNA in the mature trophozoite Q-RT-PCR (Fig. 4a). The levels of SBP in the 12 placental and 6 children's isolates analyzed (Table 1, sample group D) correlated with the levels of PF11_0505 ($R^2$ = 0.8812), corroborating the evidence of their similar temporal transcriptional profiles in 3D7, HB3, Dd2, and CS2 parasites. The placental sample Mplc345 was omitted from the normalized data analysis because it contained insufficient levels of cDNA from either normalizing gene.

In data normalized with either control gene (Fig. 4b), there was significantly more *var2csa* cDNA in the placental isolates than in the children's isolates (Table 2) (Mann-Whitney test; $P$ = 0.0043 [SBP] or 0.017 [PF11_0505]) but no significant difference in the levels of *var2csa* associated with gravidity. There was significantly more *var2csa* cDNA than endogenous control DBLγ cDNA in the placental isolates (Table 2) (Mann-Whitney test; $P$ = 0.0009 [SBP] or 0.0023 [PF11_0505]) but not in the children's isolates (Table 2) (Mann-Whitney test). The median levels of endogenous control DBLγ sequences were higher for cDNAs from the children's isolates than for those from the placental isolates, but the difference was not significant (Table 2) (Mann-Whitney test). The most abundant *var* transcript in CS2 parasites is *var2csa* (9), so the level of *var2csa* in CS2 cDNA indicates the abundance of specific *var* sequences in the cDNAs of the other samples (Fig. 4b). The abundance of *var2csa* cDNA in the child's isolate Mch1478 was apparent in the normalized data, but only a low level of *var2csa* was present in cDNA from the other child's isolate (Mch1488) in which *var2csa* was the most abundant of the analyzed *var* transcripts (Fig. 4b).

The abundance of *var-CS2* in the placental isolate Mplc78 was apparent in the normalized data (Fig. 4b). The placental

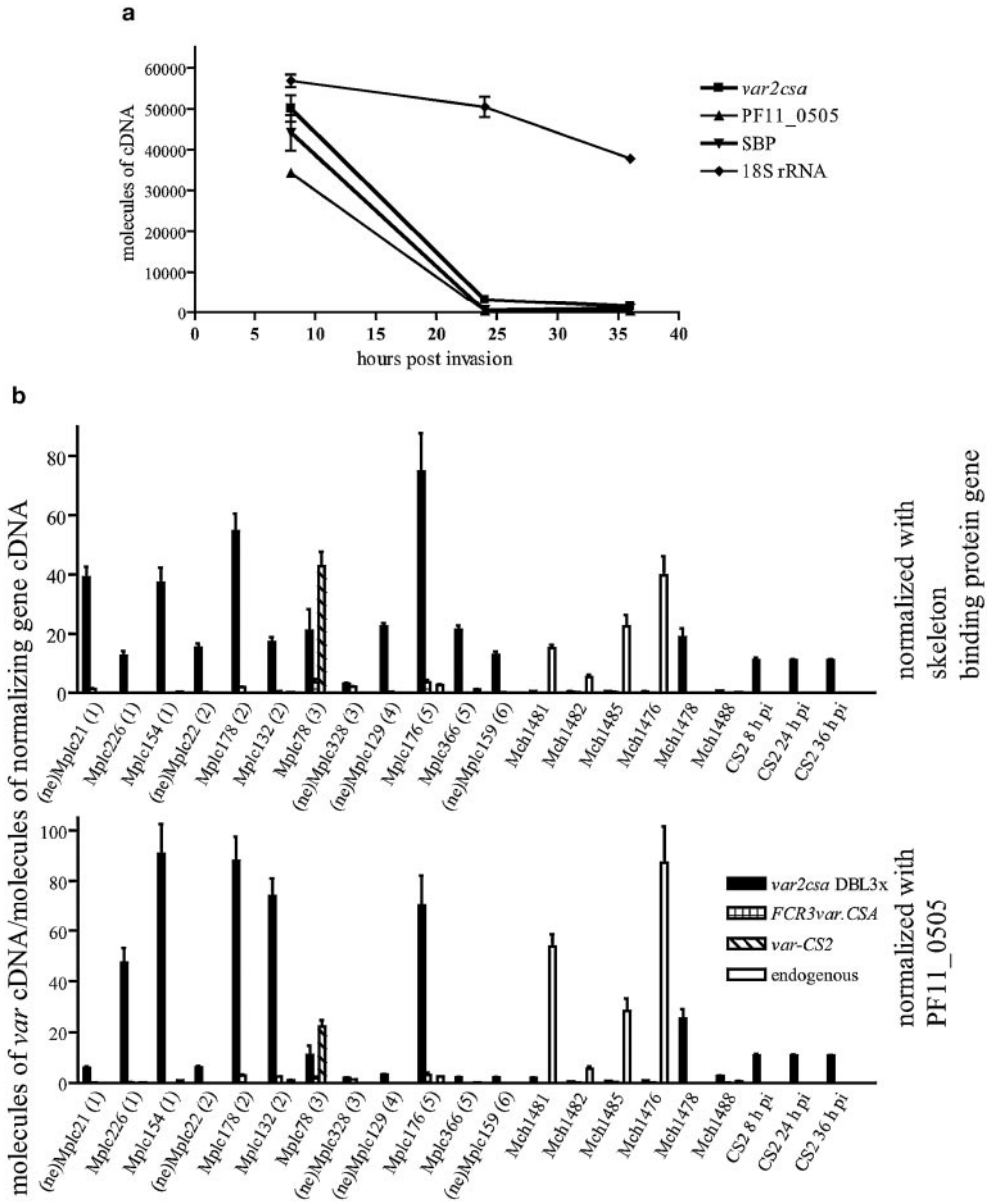


FIG. 4. (a) cDNA levels in CS2 parasites of *var2csa*, 18S rRNA, and the two genes, PF11_0505 and the SBP gene (PFE-0065w), that were used to normalize the Q-RT-PCR data. (b) Absolute quantities of *var* cDNA levels in placental (Mplc) and children's (Mch) isolates were determined by Q-RT-PCR, using standard curves, and the data were then normalized using the levels of SBP and PF11_0505 cDNAs. Consequently, the data shown represent the cDNA levels of specific *var* genes in equivalent amounts of total *var* cDNA from each isolate, as estimated using two control genes that approximate the transcriptional profile of *var* genes. The gravidity of the patient from whom each placental isolate was obtained is indicated in parentheses after the title of the isolate. Endogenous refers to the endogenous DBLγ sequence that was amplified from the sample being analyzed. The prefix "(ne)-" indicates that no endogenous DBLγ sequence control was available for that sample.

isolate Mplc328 contained similar but low normalized levels of *var2csa* DBL3x and *FCR3var.CSA* DBL3γ cDNAs (Fig. 4b). The median values for *FCR3var.CSA* DBL3γ sequences were higher for placental cDNAs than for children's cDNAs, but the difference was not significant (Table 2) (Mann-Whitney test).

## DISCUSSION

Our degenerate primer RT-PCR analysis revealed a similar diversity of transcribed DBLγ domains in placental isolates to that reported in previous studies (15, 22, 23, 32) but also revealed that *var2csa* was transcribed in all 10 placental isolates from which D3F and D3R1/D3R1.2 could amplify a product (Table 1, sample group A). This apparent dominance of *var2csa* DBL3x domains over DBLγ domains in the mRNAs of placental isolates was corroborated by the evidence of a low bias of primers D3F and D3R1 towards amplification of *var2csa* from 3D7 gDNA and by their ability to amplify *var2csa* from the cDNAs but not the gDNAs of the four placental isolates tested (Table 1, sample group A).

TABLE 2. Absolute quantitation of *var* gene sequences by Q-RT-PCR

| Gene used for normalization | Molecules of *var* gene/molecules of normalizing gene (median [interquartile range]) | | | | | |
|---|---|---|---|---|---|---|
| | *var2csa* DBL3x | | Endogenous DBLγ control | | *FCR3var.CSA* DBL3γ | |
| | Placental cDNAs | cDNAs from children's isolates | Placental cDNAs | cDNAs from children's isolates | Placental cDNAs | cDNAs from children's isolates |
| sbp | 20 (14–38) | 0.5 (0.4–10) | 0.3 (0.03–1.8) | 10 (0.06–31) | 0.7 (0.1–3.5) | 0.06 (0–0.3) |
| PF11_0505 | 7.9 (2.6–72) | 1.4 (0.5–14) | 0.6 (0.07–1.7) | 17 (0.3–71) | 0.6 (0.08–6.7) | 0.3 (0–0.7) |

Further quantitative analysis revealed that *var2csa* was the most abundant of the analyzed *var* transcripts in 12 of the 13 placental isolates examined (Table 1, sample group D), supporting the association between *var2csa* transcripts and parasites sequestered in the placenta (35, 40). The significantly higher levels of *var2csa* than of endogenous DBLγ sequences in the placental cDNAs suggested that the high levels of *var2csa* cDNA in these placental isolates did not simply reflect high levels of total *var* gene cDNA. This conclusion was supported by the lower levels of endogenous DBLγ sequences in the placental than in the children's isolate cDNAs. Although this difference was not significant, it suggested that, if anything, there was less transcription of *var* genes (other than *var2csa*) in the placental than in the children's isolates. It is quite possible that the two children's isolates in which *var2csa* was the most abundant of the analyzed *var* transcripts expressed other *var* genes lacking DBLγ domains at higher levels than *var2csa*. We did not look specifically for these, but the level of *var2csa* remained high in the normalized data for one of these children (Mch1478), indicating that *var2csa* can be an abundant transcript in nonpregnant individuals. Only low levels of *var2csa* transcripts have been described previously for nonpregnant individuals (35, 40).

The *var-CS2* DBL2γ domain was the only *var* transcript tested that was present at higher levels than *var2csa* in a placental isolate, suggesting that it may play a role in placental sequestration. Interestingly, parasites with disrupted *var2csa* that bind bovine trachea CSA in vitro also transcribe *var-CS2* at high levels (10), strengthening previous associations between transcription of this gene and adhesion to CSA (29, 30). The similar but low levels of *FCR3var.CSA* DBL3γ and *var2csa* sequences in the cDNA of the placental isolate Mplc328 suggest the existence of an abundant, unidentified *var* gene transcript in this isolate rather than indicating a role for *FCR3var.CSA* DBL3γ in placental sequestration of this isolate. Although there were higher normalized levels of the *FCR3var.CSA* DBL3γ sequence in cDNAs from mature placental parasites than in those from ring-stage parasites infecting children, the difference was not significant, and this probably simply reflects the atypically late peak of *FCR3var.CSA* transcription (25).

A role in placental sequestration cannot be excluded for PfEMP1s carrying domains homologous to the 3D7chr5var DBL3γ/720 and *FCR3var*.CSA DBL5γ domains that were transcribed by the Rplc series of placental cDNAs (Fig. 2). Like all other domains implicated in pregnancy-associated malaria, the *FCR3var.CSA* DBL5γ domain, which is homologous to 3D7chr5var DBL5γ, was previously amplified from placental isolate cDNA (15, 32). However, this domain is part of an unusually conserved subset of *var* genes (34) that are frequently transcribed by parasites infecting nonpregnant individuals (42), and unlike all other domains implicated in pregnancy-associated malaria, recombinant proteins derived from *FCR3var.CSA* DBL5γ do not bind CSA (6, 18, 19). These sequences were amplified from the Rplc placental cDNAs that had also previously been used to survey DBLα domain transcription (32). Unfortunately, there was insufficient material available from the Rplc samples to quantitate these sequences by Q-RT-PCR.

We concluded that *var2csa* was probably the principal *var* gene transcribed by parasites sequestered in the placentas of most of the participants in this study. However, the abundance of *var-CS2* in one placental isolate suggests that parasites transcribing *var* genes other than *var2csa* can be sequestered in the placenta. Larger studies must determine whether *var2csa* is the dominant *var* transcript in placental isolates from around the globe if *var2csa* is to be explored as a possible candidate for vaccination against malaria during pregnancy. Also, note the diversity within the small sample of *var2csa* DBL3x sequences isolated from one locale and examined in this study. This indicates that it may be difficult to develop a vaccine from a single *var2csa* DBL3x sequence, and thus it is essential to identify the most conserved regions of *var2csa* that elicit functional immunity.

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