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The RESA-2 Gene of *Plasmodium falciparum* Is Transcribed in Several Independent Isolates

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The relevance of the ring-infected erythrocyte surface antigen (RESA) of *Plasmodium falciparum* as a malaria vaccine candidate has been questioned of late because RESA-deficient parasites have been found to multiply normally in culture or in monkeys. The RESA-2 gene was recently described as a pseudogene highly homologous to RESA. In this report, we demonstrate that RESA-2 is not a pseudogene, because we were able to detect RESA-2 transcripts in asexual blood stages of multiple isolates by using polymerase chain reaction on reverse-transcribed mRNA. Transcription of RESA-2 was observed whether or not the isolates studied expressed the RESA protein.

Many *Plasmodium falciparum* antigens have been identified and characterized in recent years; a few of them have been considered potential candidates for the development of a malaria vaccine (reviewed in references 13 and 16). Among these is the ring-infected erythrocyte surface antigen (RESA), encoded by one of the first genes cloned from *P. falciparum* (5, 6, 10). The protein is excreted from the parasite shortly after invasion and associates with the erythrocyte cytoskeleton (11). RESA-specific antibodies efficiently inhibit in vitro parasite growth (19), and active or passive immunization protects *Aotus* monkeys against challenge (1, 4). However, both the relevance as a prime target of protective immunity and the requirement of the protein for the invasion process have been recently questioned, because it has been shown that parasite lines which do not express RESA, as a result of gene inactivation, can develop properly in vitro (3, 18) or in vivo in *Saimiri* monkeys (9). The viability of RESA-deficient lines may be explained by the provision of a RESA-like function by some other closely related molecule. Recently, Cappai et al. (2) described the presence of a gene highly homologous to RESA in the genome of *P. falciparum*. This gene was called RESA-2. We hypothesized that RESA-deficient parasites might compensate for their lack of RESA protein by expressing the RESA-2 gene. In this report, we show that the RESA-2 gene, previously reported to be silent, is transcribed in mature mRNA in several independent isolates cultivated in vitro or collected from infected *Saimiri* monkeys. Transcription was detected in the Palo Alto parasites, a RESA-deficient strain (9). Interestingly, transcription was also observed in strains that possess a wild-type RESA gene and express the Pf155/RESA protein.

MATERIALS AND METHODS

Parasites. FFG/IPC4 (RESA$^+$) parasites were obtained directly from a patient hospitalized in Cayenne, French Guiana, for severe malaria. This isolate is not infectious for the *Saimiri* monkey but multiplies well in vitro in human erythrocytes. FUP/SP (RESA$^-$) and FUP/CP (RESA$^+$) are two lines which were derived from an original Palo Alto isolate obtained in 1978. They are genetically quite distinct, differing at all loci investigated so far, i.e., Pf11. 1, SAg, MSA1, MSA2, RESA, PfPArep20, and telomere markers (7). The genetic characteristics of these parasites as well as their infectivity for splenectomized *Saimiri* monkeys have been fully detailed elsewhere (7-9).

Primers and PCR analysis. Two sets of primers were synthesized as shown in Fig. 1. Oligonucleotides 1 and 2 were selected in order to specifically amplify the RESA-2 sequence and to not cross-hybridize with RESA. Conversely, primers 3 and 4 were deliberately chosen in regions highly conserved between RESA and RESA-2 genes in order to simultaneously amplify both sequences. The sequences of the oligonucleotides used to prime the reactions are as follows: P1, 5'AAATATTATGTTCTAAGCGCTTCC3'; P2, 5'AGAATTCAAGTATGGCTTTATTGATAAT3'; P3, 5'A GAATTCTGATGCCGTAAAAGATGGTG3'; P4, 5'TGAA TTCAATATCTCCTAAAACTTGGTA3'. *Eco*RI restriction sites introduced in the primers are underlined. Polymerase chain reaction (PCR) experiments were performed by using 50 μl of a mixture containing 0.2 mM deoxynucleoside triphosphate (dNTP), sense and antisense oligonucleotide primers (2 μM each), 2 U of *Taq* DNA polymerase (Amersham) in appropriate buffer, and genomic DNA or cDNA as templates. Samples were subjected to 35 rounds of amplification.

Nucleic acid preparation and reverse transcription. Genomic DNA was prepared according to standard procedure, as previously described (12). Total RNA was extracted directly from infected erythrocytes by the guanidium-hot phenol method (15). About 10 μg of this preparation was used for reverse transcription, either directly or after treatment with RNase-free DNase (Sigma), in order to reduce genomic contamination. After inactivation of the DNase by phenol-chloroform, the nucleic acids were precipitated with ethanol. For single-strand cDNA synthesis, RNA samples were incubated for 1 h at 37°C in a reaction mixture containing 1 mM dNTP, 0.5 U of RNase inhibitor (RNasin; Promega) ml^{-1}, 20 U of avian myeloblastosis virus reverse transcriptase (Pharmacia), and 200 ng of specific antisense oligonucleotide primer P4 and were finally incubated for 5 min at 100°C. A 2- to 4-μl aliquot was used for subsequent PCR amplification. For in situ reverse transcription, infected erythrocytes were first lysed with 0.05% saponin for 10 min. Free parasites were pelleted by centrifugation at 5,000 × *g*

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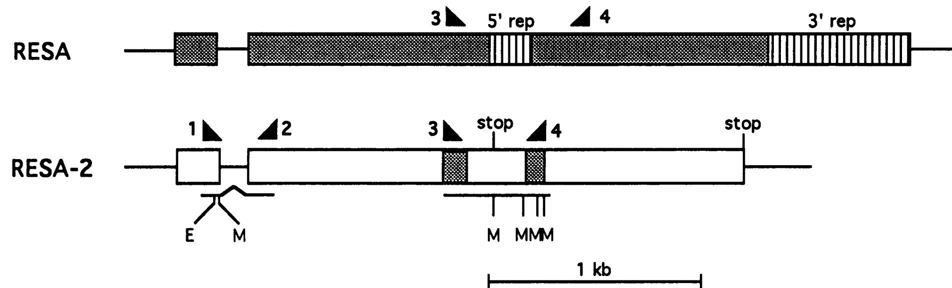

FIG. 1. Schematic representation of the RESA and RESA-2 genes deduced from FC27 (10) and FCR3 sequences (2), respectively. The coding regions are boxed. The RESA-2 gene regions which are highly homologous to RESA are shaded. The locations of the oligonucleotide primers used for the PCR and mRNA reverse transcription are indicated by arrows. The position of the in-frame stop codon and the localization of *Mse*I (M) and *Eco*RI (E) sites which are internal to the target sequences are also represented.

for 15 min, washed twice in water, and resuspended in water containing 0.5 U of RNase inhibitor ml^{-1}. Nonidet P-40 was added to a final concentration of 0.5% (total volume, 10 μl). After treatment for 5 min at 4°C, the preparation was centrifuged for 5 min at 5,000 × *g* in an Eppendorf centrifuge. The resulting supernatant was directly used for reverse transcription as described above.

Cloning and sequencing. PCR products were purified from agarose gels with Geneclean (Boehringer) as recommended by the manufacturer, restricted with *Eco*RI, and cloned in the appropriate restriction site of the M13 bacteriophage. Positive plaques were selected by hybridization with a radiolabeled probe corresponding to the genomic RESA-2 sequence, amplified with primers P1 and P2 from FUP/CP parasites. The single-strand DNA was sequenced with the Sequenase kit (U.S. Biochemicals).

Nucleotide sequence accession numbers. Nucleotide sequence data reported in this article have been submitted to the GenBank data base with the accession numbers L12976, L12977, L12978, and L12979.

RESULTS AND DISCUSSION

The aim of this study was to investigate the possibility that the RESA-2 molecule might complement the absence of RESA in RESA-deficient parasites. At first sight, this possibility seemed to be unlikely because Cappai et al. failed to detect any RESA-2 transcript by Northern (RNA) blotting, at least in the clones LF4 and 3D7 (2). Cappai et al. concluded that RESA-2 is a pseudogene. An additional argument was the presence of a single in-frame stop codon located in the middle of the coding region (Fig. 1). For that reason, we first focused our attention on the RESA-2 region predicted to contain the stop codon in three different parasite lines expressing or not expressing RESA. FUP/CP and IPC4 parasites (RESA$^+$) possess a wild-type RESA gene and express the 155-kDa RESA protein (9). The FUP/SP line (RESA$^-$) has a deletion of the RESA gene (9). The three lines were examined by combining genomic amplification and restriction analysis. In order to coamplify RESA and RESA-2 sequences, and to differentiate the PCR products by their size, we used primers P3 and P4, selected in conserved regions at the ends of the central block of repeats found in the RESA gene but absent from the RESA-2 sequence (Fig. 1). Figure 2A shows the results of a typical experiment with FUP/CP (lane CP), FUP/SP (lane SP), and FFG/IPC4 (lane IPC4) DNA. The 570-bp fragment visualized on the gel resulted from the amplification of the RESA sequence, whereas the 460-bp product was derived from the RESA-2 sequence. A single 460-bp fragment is visible in FUP/SP parasites which lack the RESA sequence (9). The in-frame stop codon found in the RESA-2 sequence at position 1864 in the sequence of Cappai et al. (2) is internal to an *Mse*I restriction site (TTAA) (Fig. 1). We took advantage of this particularity and used enzymatic digestion to check for the presence of a TAA sequence at a similar position in the RESA-2-amplified fragments. As shown in Fig. 2B, the digestion of the 460-bp purified fragments with *Mse*I was complete and gave three detectable fragments with sizes of about 220, 140, and 60 bp. The smallest remaining fragment (about 40 bp) could not be visualized on the gel because the level of sensitivity of the ethidium bromide staining was too low. From the sizes of the two largest restriction fragments visualized on the gel, we deduced that the RESA-2 sequences are likely to contain the stop codon, because the positions of *Mse*I sites are located in the positions predicted from the published sequence of the gene (2).

Next, we studied the parasites for RESA-2 RNA transcripts with Northern blotting but failed to detect mRNA by this method (not shown). In order to investigate gene expression to a closer degree, we decided to apply the reverse PCR approach, which was expected to be much more sensitive. We chose RESA-2-specific primers P1 and P2, which do not cross-hybridize with RESA sequences. They were selected in regions flanking the intron, allowing an easy identification between fragments amplified from cDNA copies and those derived from genomic DNA templates (Fig. 1). We tested various technical approaches for RNA preparation and reverse transcription before succeeding in routinely

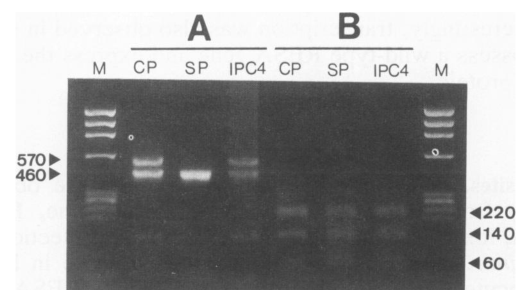

FIG. 2. Size analysis of PCR products generated with oligonucleotides P3 and P4 designed to prime RESA and RESA-2 sequences, as detailed in Materials and Methods. (A) Before digestion. (B) After *Mse*I digestion of the purified 460-bp fragments derived from RESA-2 sequences with amplification. The markers (M [in base pairs]) on each side are *Hae*III-digested PhiX 174 DNA.

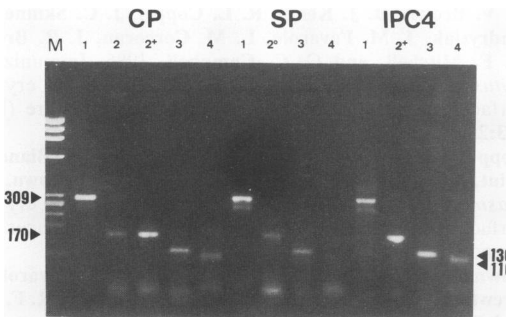

FIG. 3. Analysis of PCR products generated with primers P1 and P2 located at boundaries of the RESA2 intron as performed with either genomic DNA (lanes 1) or RT-RNA (lanes 2, 2^+, 2°) as templates. Total FUP/CP RNA was prepared by the guanidium method and was used directly for the reverse transcription step (lane 2) or had been previously subjected to DNase treatment (lane 2^+). IPC4 RNA was treated with DNase as for FUP/CP RNA (lane 2^+); SP mRNA templates were reversed in situ without additional treatment (lane 2°). The reverse transcription step is fully detailed in Materials and Methods. The various PCR products shown in lanes 2^+ and 2° were subsequently digested with *Eco*RI (lanes 3) or *Mse*I (lanes 4) before size fractionation. M, markers (in base pairs).

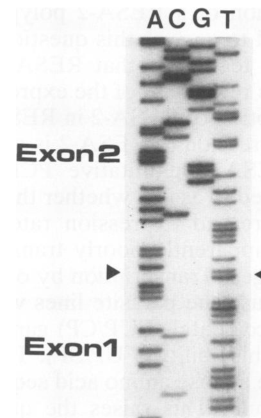

FIG. 4. Sequencing of RESA-2 RT-RNA-derived FUP/CP parasites in the region surrounding the splicing site (arrowheads).

obtaining cDNA templates suitable for PCR amplification. Even when carefully performed, the guanidium method yielded poor amounts of total RNA and always ended in coamplification of genomic DNA, as shown in Fig. 3, lane 2, for CP parasites. Treatment with DNase before the reverse transcription step reduced DNA amplification to an undetectable level (compare lanes 2^+ for CP and IPC4 with lane 2 for CP in Fig. 3). Obviously, the reversion step is critical for reproducible amplification of cDNA and requires stable mRNA templates. Thus, to reduce RNA manipulation, deleterious for molecule stability, we investigated the possibility of first-strand cDNA synthesis in situ without prior RNA extraction. In our study, this method proved to be easier to perform and much more reproducible than others (lane 2°, Fig. 3). Figure 3 compares the sizes of the PCR products resulting from amplification of the 5′ end of the gene with either genomic DNA (lanes 1) or reverse-transcribed RNA (RT-RNA, lanes 2, 2°, and 2^+) as templates. Ethidium bromide staining failed to reveal size polymorphism between the samples examined. Genomic amplification yielded a 309-bp fragment, whereas the RT-RNA-derived PCR product was a 170-bp fragment, which is the predicted size of the spliced RESA-2 sequence (2). The reverse PCR described here does not reflect the actual quantity of RESA-2 mRNA present in the developmental stages that express the gene. The different intensities of the PCR bands shown in lanes 2, 2^+, and 2° do not necessarily correlate with the rate of RESA-2 expression in different isolates. Also, the parasites used to prepare mRNA were asynchronous. cDNA-derived fragments were further examined by digestion with *Eco*RI and *Mse*I (Fig. 3, lanes 3 and 4, respectively). The restriction patterns visualized were similar in all preparations and were entirely consistent with the theoretical restriction map of a spliced RESA-2 cDNA.

Finally, the fine characterization of the products resulting from reverse PCR amplification was performed by cloning the fragments in M13 vectors and sequencing. The sequence obtained from several independent bacteriophages carrying FUP/SP or FUP/CP sequences was strictly similar to that reported for the FCR3 gene (2). An example of such an analysis is shown in Fig. 4. The sequence shows that the intron has been spliced. The exon boundaries are indicated by arrowheads. The positions of the splicing sites as well as the surrounding sequences in exons 1 and 2 are in good agreement with the data of Cappai et al. (2). A few nucleotide changes were observed in IPC4-derived RESA-2 fragments. Most templates had changes at positions 677 (T→C) and 727 (G→A), the second one resulting in an amino acid change of Y for C. Three changes were observed for a minority of templates at positions 516 (T→C), 528 (T→C), and 725 (A→G), resulting in two amino acid changes of P for L and S for L, respectively, at positions 516 and 528 (Fig. 5). The above numbers refer to the nucleotide positions in the FCR3 RESA-2 sequence, with the FCR3 nucleotide or amino acid listed first (2). The differences observed between various IPC4 templates are likely to indicate heterogeneity of the parasite population examined. Indeed, wild isolates frequently consist of several genetically distinct subpopulations. Because the mutation at position 528 affects the internal *Mse*I restriction site, and because digestion of IPC4 cDNA-derived fragments was apparently total on the gel (Fig. 3, lane 4), we deduced that the type 2 IPC4 subpopulation was very poorly represented in the total IPC4 population.

The isolation and characterization of RESA-2 transcripts in several isolates indicate that RESA-2 is not a pseudogene and that the synthesis of a RESA-2 protein in *P. falciparum* is highly probable. Very often, pseudogenes accumulate several deleterious mutations. Such deleterious mutations were not observed in the RESA-2 sequences examined, which conversely appeared well conserved from one isolate to another. As previously reported for FCR3 parasites, we were able to detect the presence of a TAA in-frame stop codon in the DNAs examined. Does it represent the true termination codon of the gene, which would then result in a significantly smaller protein than RESA? The identification

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FCR3   ILYIILNYNFTYSGNSTCRLQFTHRCSRNLYGKELSIKPYL
FUPCP  -----------------------------------------
FUPSP  -----------------------------------------
IPC4/1 -------------------------Y---------------
IPC4/2 -P---S-----------------------------------
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FIG. 5. Translated partial coding sequences of *P. falciparum* RESA-2 mRNA from FUP/CP, FUP/SP, and IPC4 subpopulations 1 and 2 and comparison with the previously described FCR3 sequence (2). Only amino acid differences (boldface) are shown.

and characterization of a RESA-2 polypeptide in *P. falciparum* are needed to answer this question.

One noticeable feature is that RESA-2 mRNA was detected in parasites regardless of the expression of the RESA protein. Transcription of RESA-2 in RESA-expressing lines indicates that expression of RESA-2 is not contingent upon extinction of RESA. Quantitative PCR on synchronous parasites is required to assess whether the absence of RESA results in an increased expression rate of RESA-2. The RESA-2 gene is apparently poorly transcribed, at least by asexual blood stages. Transcription by other stages was not investigated because the parasite lines we studied are low-level (IPC4) or accidental (FUP/CP) gametocyte producers or had even lost this ability (FUP/SP). The RESA-2 protein was found to have a close amino acid sequence homology to the RESA molecule. This raises the question of whether RESA-2 can substitute for a RESA function in RESA-deficient parasites. The low level of RESA-2 transcription may argue against this possibility, but, on the other hand, until the function of the RESA protein is clarified, it is difficult to predict the amount of protein actually required to perform a RESA function in *P. falciparum* parasites.

Though RESA and RESA-2 are located on different chromosomes (chromosomes 1 and 11, respectively), they obviously belong to the same gene family. Interestingly, in recent years, several duplicated genes have been discovered in *P. falciparum*. In all cases reported so far (HRPII/HRPIII [20], SERP/SERP-H [14], GBP/GBP-H [17]), both members are actively transcribed, sometimes at different rates (17), by blood stages. The results reported here indicate that this also holds true for RESA and RESA-2. Homologous proteins in *P. falciparum* have been suggested to play a role in evasion of the host immune response (17). We would like to point out that the presence of two analogous functional genes may also offer a selective advantage because mutation and/or deletion of one of the copies is predicted to have less severe phenotypic consequences as long as the other gene remains expressed. The apparently normal behavior of RESA-deficient parasites (in vitro or in vivo) supports this hypothesis.

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