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Transcription of the gene for the merozoite surface antigen MSA2 of the human malaria parasite *Plasmodium falciparum* during the asexual cycle

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The transcription of the *Plasmodium falciparum* gene for the MSA2 antigen has been studied throughout the parasite's asexual growth cycle. For this purpose poly(A)$^+$ RNA from different times of the cycle was tested for the presence of the mRNA encoding MSA2 by in vitro translation and subsequent analysis of the translation products by immunoprecipitation with an antibody against MSA2. The results revealed that this mRNA is present in trophozoites, reaches the highest concentration during the transition from the trophozoite into the schizont stage, and persists until the cycle end. Minute amounts of this mRNA were also detected in rings. In addition, the data confirmed that the primary translation product is not proteolytically processed at any time of the cycle.

Human malaria; Merozoite surface protein; Translation in vitro; Transcription; *Plasmodium falciparum*

1. INTRODUCTION

The surface proteins of *Plasmodium falciparum* merozoites play a crucial role during malarial infection since they participate in the invasion of the erythrocytes and are exposed to the host immune system during merozoite release and invasion. The merozoite surface protein MSA2 is a polymorphic antigen with an apparent relative molecular weight (M_r) varying among different strains; this antigen is being considered for the development of a malaria vaccine because it is expressed on the parasite surface and antibodies against it block the invasion [1–11]. MSA2 has been demonstrated in trophozoites, schizonts, segmenters and isolated merozoites by several techniques: using either poly- or monoclonal antibodies the protein was detected by immunoblotting [1,7] and immunoprecipitation of extracts from synchronous cultures [6,9], by immuno-EM [6], and by IFA [1,9]. In the ring stages low MSA2 levels could be detected by immunoprecipitation [6] and immunoblotting [9] of extracts from synchronous cultures using polyclonal antibodies. However, no antigen could be detected in rings by using single monoclonal antibodies in IFA [1], immunoblotting of extracts from ring cultures [7], or immunoprecipitation of proteins pulse-labelled before the invasion and isolated from ring stages [1].

The MSA2 gene from several strains has been sequenced and the comparison of the deduced amino acid sequences has revealed a good correlation between the degrees of serological and structural divergence among these strains [2,12]. On the other hand, little is known about the transcription of this gene during the asexual cycle; the presence of MSA2-RNA has only been demonstrated, by Northern analysis, in a mixed population of trophozoites and schizonts [2].

Several observations have demonstrated that *P. falciparum* mRNA directs, in the reticulocyte cell-free translation system, the synthesis of the specific *P. falciparum* proteins it codes for. Firstly, the translation products are very similar to the parasite proteins made in vivo (results obtained by using both mRNA isolated from asynchronous cultures [13] and mRNA isolated from the tropozoite or from the schizont stage [14]). Secondly, the translation products of mRNA from asynchronous cultures of independent isolates are virtually identical [14]. Thirdly, human and monkey immune sera react specifically with polypeptides synthesized in vitro [13–16]. For these reasons, it is plausible to detect the mRNA encoding a given parasite protein by in vitro translating the parasite mRNA, and subsequently by investigating the presence of the protein among the translation products with a specific antibody. This approach was employed here to study the transcription of the MSA2 gene in the strain FCB1 during the asexual erythrocytic cycle. The FBC1-MSA2 antigen, detected on the outer merozoite surface by surface-iodination [17,18], is a glycoprotein with an M_r of

Abbreviations: (buffers used for immunoprecipitation) T, 50 mM Tris·Cl (pH 8.0); E, 5 mM EDTA (pH 8.0); N, 0.5 M NaCl; Z, 5 mM Zwittergent 3-12 (Calbiochem-Behring); A, 1 mg/ml bovine serum albumin; i, mix of protease inhibitors (2% aprotinin (Bayer); antipain, bestatin and pepstatin A (Sigma), each at 1 μg/ml; 2 mM phenylmethylsulphonyl fluoride (BDH)).

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46K, recognized by human immune serum and able to elicit the production of antibodies that inhibit invasion of erythrocytes [6].

2. MATERIALS AND METHODS

2.1. *Culturing and synchronization of* P. falciparum

Parasites of the FCB1 strain from Colombia [17] were cultured [19] and synchronized by alternate applications of the plasmagel [20] and the sorbitol [21] procedures.

2.2. *Isolation of total RNA*

A highly synchronous *P. falciparum* culture was employed to isolate parasite RNA at 6, 13, 20, 27, 32, 37, 42 and 47 h after the merozoite invasion. For each RNA preparation a culture sample was centrifuged in the presence of vanadyl-ribonucleoside complexes (BRL). The pellet was resuspended in 8 ml of guanidinium lysis buffer (4 M guanidinium isothiocyanate, 50 mM Tris·Cl (pH 7.5), 10 mM EDTA, 2% Sarkosyl, 1% 2-mercaptoethanol) and transferred into a 50-ml tube. While maintaining the tube in a 60°C bath, the suspension was mixed with 8 ml of melted phenol, passed 10 times through a syringe fitted with an 1.5-mm-diameter needle, mixed with 8 ml of acetate-Tris-EDTA buffer (100 mM sodium acetate (pH 5.2), 10 mM Tris·Cl (pH 7.4), 1 mM EDTA (pH 8.0)) and 24 ml of chloroform/isoamyl alcohol (24:1), and shaken for 10 min by hand. The tube was then cooled in an ice-water bath and centrifuged to separate the phases. The RNA was then re-extracted, once with equal parts of phenol and chloroform and twice with chloroform. Extractions were carried out by hand. The last aqueous phase was mixed with 2 vols of ethanol and stored at −70°C.

2.3. *Isolation of poly(A)⁺ RNA*

Stage-specific poly(A)⁺ RNA was isolated from each preparation of total RNA by affinity chromatography with oligo(dT)-cellulose. For this purpose the RNA (conserved in ethanol) was pelleted, dried, dissolved in 500 μl of diethylpyrocarbonate-treated bidistilled H_2O, heated to 65°C for 3 min, quickly cooled on ice, mixed with 1 vol of 2 × binding buffer (1 × binding buffer is 0.5% SDS, 50 mM Tris·Cl (pH 7.5), 500 mM LiCl) and added to oligo(dT)-cellulose (Type 3, Collaborative Research) previously washed with binding buffer and kept in a minimal volume. The suspension was stirred slowly for 2 h, transferred into a column, and the flow-through was collected, heated, cooled and passed through the column three times. The column was then washed with 10 column-vols of binding buffer, followed by 5 column-vols of wash buffer (50 mM Tris·Cl (pH 7.5), 500 mM LiCl). The poly(A)⁺ RNA was eluted with 2–3 column-vols of 10 mM Tris·Cl (pH 7.5). The OD_{260} absorbing fractions were pooled, the RNA was precipitated with ethanol and resuspended at 1 μg/ml in diethylpyrocarbonate-treated H_2O. By this procedure the amount of contaminant rRNA, as judged by agarose gel electrophoresis, was extremely low (not shown), and 10.77 ± 2.61% of the original total RNA was recovered as poly(A)⁺ RNA (calculated from the OD_{260} data for the eight preparations).

2.4. *In vitro translation*

The Rabbit Reticulocyte Lysate Translation System from New England Nuclear was used as indicated by the manufacturer, except that the assay had a final volume of 25 μl, contained 40 μCi of [^{35}S]methionine, and was incubated at 37°C for 120 min. The translation mix contained 4 μg/ml of poly(A)⁺ RNA (in preliminary experiments the maximum incorporation rate of radioactivity into TCA-precipitable material and the highest number of polypeptide bands were obtained at this concentration (not shown)). To analyse the translation products, a 5 μl aliquot of the mix was solubilized in 50 μl of sample buffer [22] and heated at 90°C for 10 min. The denatured ^{35}S-labelled polypeptides were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 5–20% gradient gel under reducing conditions, and visualized by fluorography [17]. In a parallel series of experiments, after the translation the whole mix (25 μl) was subjected to immunoprecipitation (see below). In order to visualize the incorporation directed by the endogenous RNA of the translation system, a background translation assay was included, in which *P. falciparum* poly(A)⁺ RNA had been omitted.

2.5. *Immunoprecipitation*

The 25-μl translation mix received sequentially 5 μl of 30 mM Zwittergent 3-12 (Calbiochem-Behring), 90 μl of buffer TEi, 5 μl of a rabbit antiserum raised against purified MSA2 [6], and it was incubated overnight at 0°C. Thereafter, 60 μl of a suspension of protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) was added (the Protein A-Sepharose had been previously swollen and washed two times in TENZAi, washed two times more in TEZi, and suspended in 1 vol of TEZi). The mixture was then incubated at 4°C for 3 h with vigorous shaking. The immunocomplex-containing sediment was washed 3 times with TENZAi and 3 times more with TENi. To release the precipitated antigens, the sediment was resuspended in 45 μl of 2 × sample buffer and heated at 90°C for 10 min. The radiolabelled polypeptides were analysed by SDS-PAGE followed by fluorography (see above). Negative controls for the immunoprecipitation using a non-immune rabbit serum were also included.

3. RESULTS AND DISCUSSION

3.1. *In vitro translation of stage-specific mRNAs*

Previous work has demonstrated that *P. falciparum* mRNA is translated by the rabbit reticulocyte cell-free translation system into specific *P. falciparum* proteins (see section 1). On the basis of this, stage-specific mRNAs synthesized throughout the entire 48 h long erythrocytic cycle were studied here by analysing their in vitro translation products. For this purpose poly(A)⁺ RNA was prepared from a highly synchronous parasite culture at various times after merozoite invasion: at 6, 13 and 20 h (ring stages); at 27, 32 and 37 h (trophozoite stages); and at 42 and 47 h (schizont stages). Each poly(A)⁺ RNA preparation was then translated in the reticulocyte lysate system, and the synthesized ^{35}S-labelled polypeptides were separated by SDS-PAGE and visualized by fluorography. The sharpness of the obtained bands (see Fig. 1) indicate that the translated RNAs were composed of essentially intact, full length mature mRNA molecules and contained few levels of either RNA transcript molecules mechanically sheared during the RNA isolation or partially synthesized RNA chains (mechanical shearing is reduced during the RNA isolation by omitting use of the vortex and conducting extractions by hand. Partially synthesized RNA does not belong to the intra-cellular pool of poly(A)⁺ RNA molecules since polyadenylation takes place in vivo after the RNA synthesis has finished; it is therefore not expected in a good poly(A)⁺ RNA preparation).

As shown in Fig. 1, in addition to the polypeptides encoded by the endogenous RNA of the lysate (see track −), many other polypeptides were synthesized in the presence of the parasite poly(A)⁺ RNAs. The following observations confirm the *P. falciparum*- and stage-specificity of these polypeptides. (i) Their synthesis was stimulated only in the presence of parasite RNA. (ii) A pool of immune sera from individuals living in an

endemic area recognized a great part of these polypeptides (not shown). (iii) The progression of total protein synthesis rate depicted in Fig. 1 corresponds to that described for parasite cultures [23,24]: it is low in rings and young trophozoites, increases in the middle and mature tropozoites, and remains high until parasite maturation. (iv) As the pattern of proteins synthesized in vivo is characteristic for each developmental stage, so the pattern of polypeptides synthesized in vitro is stage-specific. (v) Both the largest number of proteins and the proteins with the highest M_r are synthesized by mature parasites in culture [24–26]. This is also observed with the late poly(A)$^+$ RNAs in vitro (Fig. 1).

3.2. *Identification of the MSA2-mRNA*

To study the transcription of the MSA2 gene during the asexual cycle, the poly(A)$^+$ RNAs isolated at 13, 20, 32, 37, 42 and 47 h after invasion were translated in vitro, and the translation products were subjected to immunoprecipitation with a polyclonal antiserum raised in rabbits against MSA2. As revealed by the results shown in Fig. 2 (reproduced by an independent experiment, not shown), the major polypeptide precipitated had an M_r of 43K. This polypeptide corresponds to the nonglycosylated moiety of MSA2 for the following reasons. (i) The antibody used was specific for MSA2. (ii) As expected from the absence of glycosylation during the in vitro translation, the electrophoretic mobility of the polypeptide is slightly increased and the resulting M_r (43K) lower with respect to the glycosylated MSA2 (46K). (iii) As the natural MSA2 is one of the major schizont and merozoite proteins [6], so 43K is also among the major polypeptides encoded by late mRNAs (see in Fig. 1 tracks for mRNAs from 37, 42 and 47 h). (iv) The 43K polypeptide must contain some of those parasite epitopes that come into contact with the host immune system during the natural infection because 43K was precipitated by a pool of human immune sera, but not by a human non-immune serum (data not shown). Therefore, the presence of the 43K band in Fig. 1 (translation products) and Fig. 2 (immunoprecipitated translation products) must indicate the presence of MSA2-mRNA.

3.3. *The co-precipitated polypeptides are neither precursors nor processing fragments of 43K*

As shown in Fig. 2, the anti-MSA2 serum also precipitated low amounts of other polypeptides, four larger (159K, 84K, 62K and 50K) and four smaller (39K, 36K and a 33K doublet) than 43K. The co-precipitation implies that they contain some of the epitopes encoded by the 43K amino acid sequence (conformational epitopes are not involved because Zwittergent was used prior and during incubation with the antiserum). If any of the four large polypeptides was a precursor of 43K (primary translation product or processing intermediate), then it should comprise the complete 43K

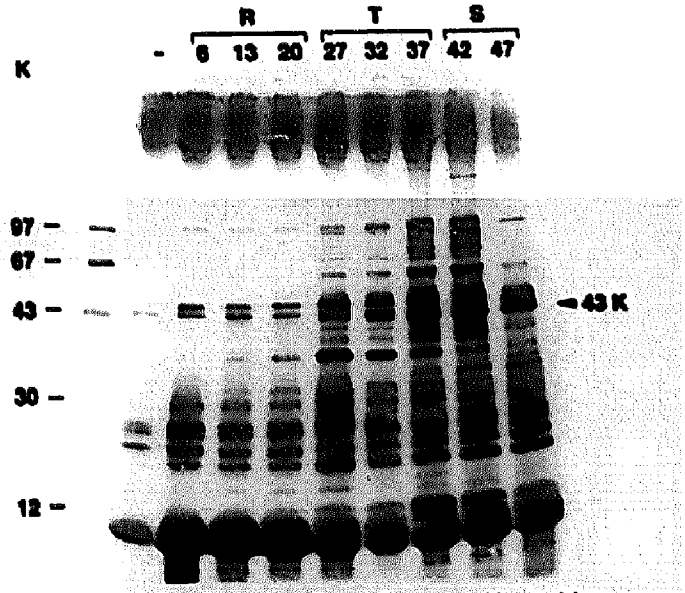

Fig. 1. In vitro translation products of stage-specific *P. falciparum* poly(A)$^+$ RNAs. Poly(A)$^+$ RNA was isolated from a synchronized parasite culture at various times of the asexual growth cycle and translated with a rabbit reticulocyte lysate. The translation products were analysed by SDS-PAGE followed by fluorography. The number above each track gives the time of the cycle (in hours after merozoite invasion) at which the poly(A)$^+$ RNA was isolated. R, rings; T, trophozoites; S, schizonts. Track –, background translation assay in which no parasite RNA was added. Molecular weight markers were used on both gel sides but are shown only on the left. On the right is indicated the position of the 43K polypeptide.

sequence, and hence, all the 43K sequence epitopes recognized by the antiserum. Consequently the antiserum would precipitate such a precursor and 43K with similar efficiencies. The extent of precipitation of a given polypeptide can be estimated by the relationship between the densities of the polypeptide band on the fluorographs of Fig. 1 (synthesized material) and Fig. 2 (precipitated material). By calculating such a relationship for the 43K band and for any of the other four large polypeptides, one finds that all four large polypeptides were precipitated to a much lesser extent than 43K; therefore they can not be 43K precursors. On the other hand, if any of the co-precipitated smaller polypeptides 39K, 36K or 33K (doublet) was a proteolytic derivative of 43K, it would consist of 91%, 84% or 77% of the 43K sequence, respectively. Such a polypeptide would thus contain enough of the 43K sequence epitopes to assure an efficient immunoprecipitation. On the contrary, the relationship between the densities for synthesized and immunoprecipitated material (fluorographs of Figs. 1 and 2, respectively) indicated that all the smaller polypeptides were precipitated with a much lower efficiency than 43K; thus, they must be distinct polypeptides. As neither precursors nor processed fragments of 43K were detected, it can be concluded that 43K is, at the same time, the primary and final translation product of MSA2 mRNA, i.e., that proteolytic cleavage of this antigen does not take place during the in vitro transla-

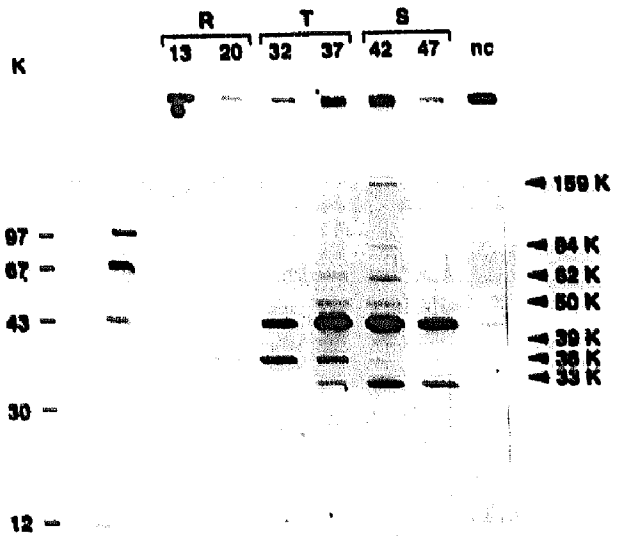

Fig. 2. Immunoprecipitation of in vitro translation products of stage-specific poly(A)+ RNAs by a MSA2 specific antiserum. The poly(A)+ RNAs isolated 13, 20, 32, 37, 42 and 47 h after invasion, were translated in vitro and the products subjected to immunoprecipitation with a polyclonal rabbit antiserum raised against purified MSA2. The precipitated polypeptides were analysed by SDS-PAGE followed by fluorography. R, rings; T, trophozoites; S, schizonts. Track nc, negative control, in which the translation products of the poly(A)+ RNA from 42 h were subjected to immunoprecipitation with a nonimmune rabbit serum. Molecular weight markers were used on both gel sides but are shown only on the left. On the right are indicated the positions of the polypeptides co-precipitated with 43K.

tion. This conclusion is consistent with reported data from pulse-chase experiments showing that MSA2 from strain FCb1 did not change its electrophoretic mobility from schizonts to merozoites, and MSA2 from strain T9/94 also remained invariable from the 35th hour to the end of the cycle [1].

The existence of epitopes common to 43K and to the co-precipitated polypeptides is not surprising, since cross-reactive epitopes are frequent among *P. falciparum* antigens [27–31].

3.4. *Time of synthesis of the MSA2-mRNA during the asexual cycle*

According to the data shown in Fig. 1, the MSA2-mRNA transcription begins in young trophozoites (around the 27th hour), increases during the maturation of trophozoites (27–37 h), reaches the highest levels during the transition from trophozoites to schizonts (around 42 h), and decreases moderately towards the end of the cycle (47 h). In addition, a residual, very low level of this mRNA can be detected in rings from 13 h, but not in rings from 20 h (see Fig. 2).

3.5. *MSA2 protein synthesis and MSA2 gene transcription*

It is known that the concentration of the MSA2 protein increases during the second half of the asexual development (trophozoites, schizonts, segmenters), reaching a maximum around the 42th hour of the cycle [1,6]. After the invasion however, the protein is not detected or is detected a low concentrations in the new ring stages (see Introduction). This last data indicates that the molecule is completely or partially lost during invasion (merozoites shed a portion of their coat during invasion [32]), and thus suggests a role for the protein in the attachment to the erythrocyte. In the present work it has been demonstrated that the transcription of the MSA2 gene follows a course that coincides with the course of synthesis of the MSA2 protein. This common pattern clearly indicates that the expression of MSA2 is controlled at the transcriptional level. The very low amount of MSA2-mRNA detected at 13 h may result from residual activity of the gene, or from residual mRNA molecules, synthesized before the invasion, that entered the new infected erythrocyte. This low mRNA level could be partially responsible for the low level of MSA2 protein detected in rings by others.

The study of the mechanisms regulating the MSA2 gene transcription can help to better understand the parasite differentiation and thus allow one to interfere with the malarial infection. In addition, the discovery of the elements controlling the transcription may help to establish a system which reproduces the natural milieu for the production of a recombinant malaria vaccine.

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