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Protein and Nucleic Acid Synthesis During Synchronized Growth of *Plasmodium falciparum*†

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***Plasmodium falciparum*, the human malarial parasite, was synchronized for asexual growth and pulse labeled to determine when RNA, protein, and DNA synthesis occurred. RNA was synthesized during two periods in the 48-h developmental cycle, protein synthesis occurred throughout the cycle, and most DNA was synthesized just before nuclear division (schizogony).**

Recent interest in developing a synthetic vaccine for malaria by using molecular biology techniques has led to the identification of many antigens produced by *Plasmodium falciparum* (for a review, see reference 13; also see references 3, 5, 8, 12, and 14). To facilitate the isolation of mRNA to produce cDNA libraries and DNA for genomic libraries, we determined when RNA and DNA are synthesized relative to antigens during the asexual intraerythrocytic development of the parasite.

P. falciparum was grown in human erythrocytes by the candle jar method of Trager and Jensen (17). Development was synchronized by first selecting for trophozoite- and schizont-infected erythrocytes (16) and culturing them for 18 to 24 h. The cultures were then treated with sorbitol to select for ring stage-infected erythrocytes (10). Usually two cycles of synchronization were used before labeling. Parasitemia (1 to 10% infected erythrocytes) and stages of development were determined by microscopic inspection of Giemsa-stained thin smears. Ring-stage trophozoites were those with a sharply stained ring-shaped cytoplasm. Mature trophozoites had cytoplasm that was solidly blue-stained but was no longer ring shaped and contained a single nucleus. Schizonts contained two or more nuclei, and segmentors were multinucleate with discernible merozoites in the erythrocyte.

Synchronized cultures were pulse labeled for eight sequential 6-h periods beginning at the ring stage of development. Cultures were split into two portions and labeled separately for proteins and nucleic acids in 0.5-ml portions. The remaining unlabeled cells were maintained with standard culture procedures until labeled. Proteins were labeled by adding L-[^{35}S]methionine (20 μCi/ml) to methionine-free medium. Nucleic acids were labeled with 2,8,5′-[^{3}H]adenosine (10 μCi/ml) in complete medium. Thin smears were Giemsa stained and examined microscopically at the beginning and end of each labeling period to determine the stages of development of the parasites. Labeled cells were collected at the end of each time period and frozen at −80°C until analyzed. Uninfected erythrocyte controls maintained under the same conditions incorporated no radioactivity.

Cultures of *P. falciparum* were labeled with adenosine because previous experiments showed that this purine is readily incorporated into both DNA and RNA in another *Plasmodium* species (11). Nucleic acids were extracted and precipitated with ethanol (4) with 100 μg of carrier yeast tRNA in 300 mM sodium acetate and then pelleted at 17,000 × *g* for 15 min. Nucleic acids were dissolved in 10 mM Tris hydrochloride (pH 7.5)–10 mM EDTA. A portion of each sample was digested with 45 μg of DNase I (bovine pancreatic, RNase free; Calbiochem-Behring) per ml in 10 mM Tris hydrochloride (pH 7.5)–10 mM $MgCl_2$ or with 1 μg of RNase A (boiled to inactivate DNase) per ml in 10 mM Tris hydrochloride (pH 5)–0.1 mM EDTA. Enzyme digests were incubated at 30°C for 90 min. The radioactivity incorporated into undigested nucleic acid after enzyme treatment was determined by precipitation in 30 volumes of ice-cold 10% trichloroacetic acid. Trichloroacetic acid-precipitable nucleic acids were collected on glass filters and counted in scintillation cocktail.

Figure 1 graphically shows a 48-h developmental cycle typical of *P. falciparum* with this growth synchronization procedure. Although synchrony was not ideal, the incorporation of ^{3}H into RNA and DNA could be correlated with the growth stages present at the end of the 6-h growth periods.

Incorporation of ^{3}H into RNA began within the first 6 h of growth but peaked during two periods in the developmental cycle (Fig. 1). The first occurred after 12 to 18 h, corresponding with the development of mature trophozoites. The second occurred after 24 to 30 h, when mature trophozoites were still present and about one-third of the parasites had undergone nuclear division (schizogony). We have not determined what proportions of the RNA synthesized during these time periods were mRNA, rRNA, and tRNA. However, 1 to 5% of the RNA isolated from 24- to 48-h-old trophozoites was retained by oligodeoxythymidylate-cellulose (1), indicating that polyadenylation had occurred. Other investigators have estimated that rRNA represents from 80% (6) to more than 90% (18) of the total RNA isolated from asynchronous cultures of *P. falciparum*. Furthermore, functional mRNA has been isolated from both ring-stage parasites (0 to 18 h) and trophozoites and schizonts (24 to 48 h) (4).

DNA synthesis began with schizogony (18 to 24 h of growth, Fig. 1), and maximum incorporation of radioactivity into DNA occurred at 30 to 36 h when multinucleate schizonts and segmentors constituted 65% of the culture. These results are similar to those of Inselburg and Banyal (7), who determined that maximum synthesis occurred at 31.5 h, although they used different conditions to synchronize growth and label cells. We confirmed that during this time ^{3}H was incorporated into parasite DNA rather than into erythrocyte components by purifying the DNA in a CsCl

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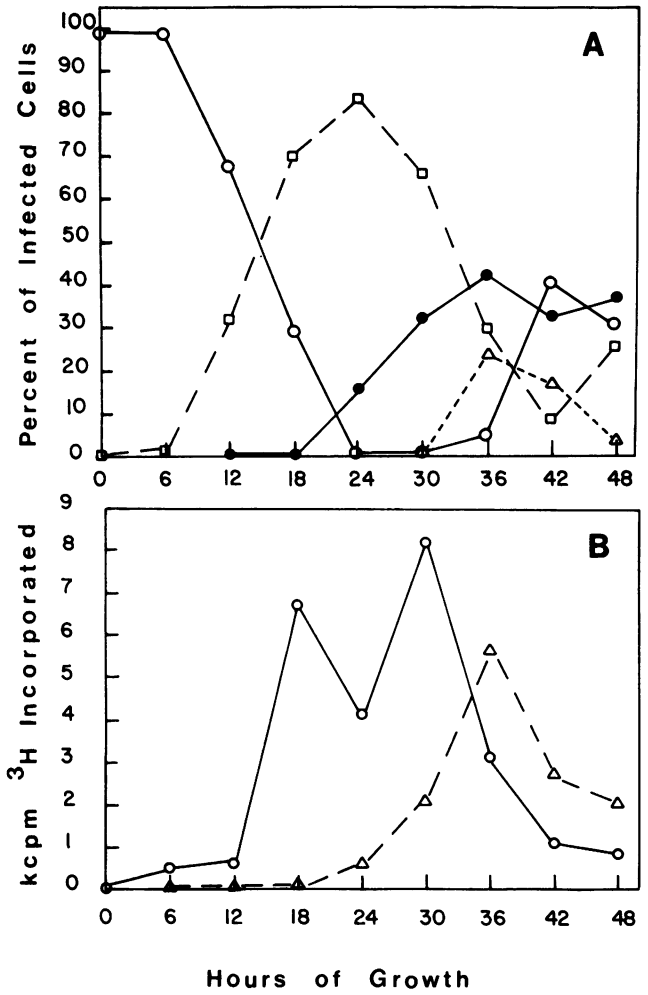

FIG. 1. Developmental stages in synchronized cultures of *P. falciparum* and ^{3}H incorporation into nucleic acids. (A) Percentages of cells infected with ring-stage parasites (○), mature trophozoites (□), schizonts (●), and segmentors (△) as determined for 100 infected erythrocytes. (B) ^{3}H incorporation into DNA (△) and RNA (○) for synchronized cells. Points represent radioactivity present in nucleic acids at the end of the 6-h labeling period. The results shown here are for the Honduras-1 strain.

(55%, wt/wt) equilibrium buoyant density gradient. A single species of [^{3}H]DNA was detected with a density of 1.678 g/cm^3, which corresponds to the 18% G+C content of *P. falciparum* DNA (15).

Similar results for RNA and DNA synthesis were obtained in three separate labeling experiments with the Honduras-1 and FVO strains (17) of *P. falciparum*.

Proteins were labeled with L-[^{35}S]methionine throughout the growth cycle. Labeled cells were lysed by adding 20 volumes of immunoprecipitation buffer (4) and then mixing and incubating them at 37°C for 10 min. Insoluble material was pelleted at 16,000 × *g* for 5 min, and the soluble proteins were separated by polyacrylamide gel electrophoresis in 11% polyacrylamide gels (9) and visualized by fluorography (2). Labeled proteins of similar relative molecular weights were synthesized throughout the developmental cycle, but at least eight new proteins were synthesized during the last 30 h of growth, representing trophozoite- and schizont-specific proteins (Fig. 2).

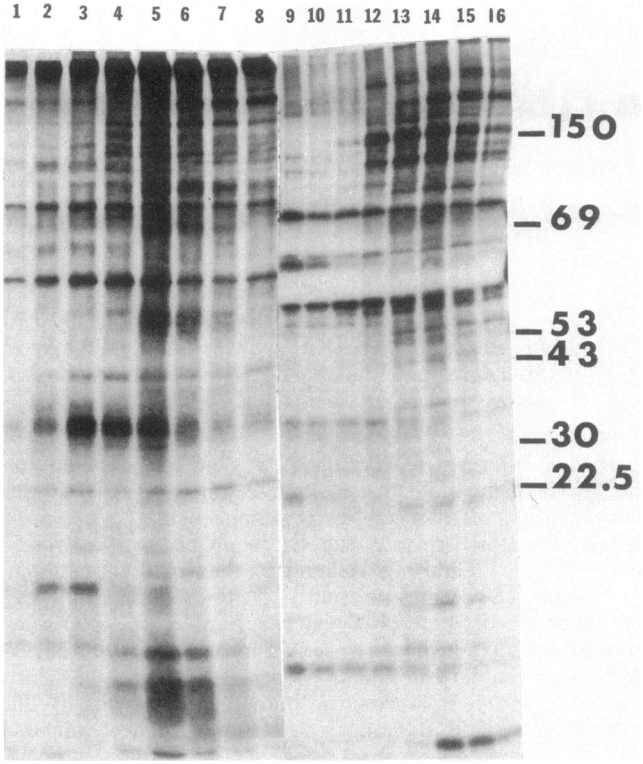

FIG. 2. ^{35}S-labeled proteins and antigens from synchronized cultures of *P. falciparum*. Lanes: 1 through 8, total labeled soluble proteins; 9 through 16, proteins immunoprecipitated with immune monkey serum for the Honduras-1 strain. The lanes correspond to the following labeling periods: 1 and 9, 0 to 6 h; 2 and 10, 6 to 12 h; 3 and 11, 12 to 18 h; 4 and 12, 18 to 24 h; 5 and 13, 24 to 30 h; 6 and 14, 30 to 36 h; 7 and 15, 36 to 42 h; 8 and 16, 42 to 48 h. The sizes of the molecular weight standards (native gamma globulin, bovine serum albumin, gamma globulin heavy chain, ovalbumin, carbonic anhydrase, and gamma globulin light chain) are indicated at the right in kilodaltons.

Parasite antigens were identified by immunoprecipitation with immune *Aotus* sp. monkey serum (4) followed by polyacrylamide gel electrophoresis and fluorography (Fig. 2). Normal monkey serum did not immunoprecipitate proteins that could be visualized under these conditions (data not shown). Most of the antigens were synthesized during the 18- to 42-h interval. The major antigens (215,000 molecular weight [215K], 200K, 185K, 175K, 170K, 155K, 145K, 139K, 117K, 115K, 100K, 95K, 60K, 53K, 21K, 12.5K, and 8.5K antigens) were similar to those obtained by other investigators (3, 5, 8, 12–14). In addition, some of the antigens had molecular weights corresponding to those of antigens synthesized by in vitro translation of RNA (4).

The results presented here were obtained with cultures of *P. falciparum* that were synchronized by enriching first for mature trophozoites, schizonts, and segmentors and then for ring-stage parasites. This procedure did not yield a perfectly synchronized culture; however, the techniques employed did not appear to affect physiologically the surviving parasites. Furthermore, these techniques can be used to enrich for particular developmental stages of the parasites in the relatively large culture volumes needed for RNA and DNA isolation. For investigations in which perfect synchrony is critical, other methods could be used (7).

Our results suggest that RNA synthesis at the mature trophozoite and schizont stages of development precedes synthesis of most of the major antigens of *P. falciparum*. By isolating mRNA from organisms at these stages, it would be possible to enrich for antigen-specifying mRNAs for cDNA cloning. Furthermore, isolating DNA from multinucleate schizonts and segmentors when most DNA synthesis has been completed should be most effective for genomic cloning.

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