The Effects of Glucose Concentration on the Reciprocal Regulation of rRNA Promoters in *Plasmodium falciparum*.

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The development of Plasmodium falciparum is remarkably sensitive to glucose concentration. We have investigated the effects of glucose concentration on the parasite development cycle as reflected by changes of ribosomal RNA (rRNA) transcription. We showed that glucose starvation differentially affects transcriptional control of the rRNA genes by sharply repressing transcription from those loci involved with asexual development of the parasite while up-regulating transcription at those loci involved with sexual development of the parasite. Temperature change also affects regulation of transcription. We found that the effects of temperature and glucose were synergistic. We identified and compared the upstream region of the transcription start sites of each gene. These putative promoter structures are considerably different from one another and contain structures remarkably similar to rRNA control elements in other organisms.

The number of ribosomes present in a cell is directly related to its protein-synthesizing activity and size of the cell. The ribosomes may make up 45% of the dry weight of an actively growing cell; thus, control of the synthesis of the molecules that make up the ribosome (e.g. rRNA) is essential to balance the needs of the cell with its external environment. Control of growth and function of a cell by regulation of ribosome production is somewhat analogous to controlling a vehicle with accelerator and brakes. Speed has to be monitored and regulated in conjunction with driving conditions for the sake of both fuel efficiency and safety. Likewise, the organism must have the means to speed up, slow down, or remain constant developmentally in response to its changing environment. Any one of a variety of metabolic rates may have an adaptive advantage depending on the situation.

Although Plasmodium is a eukaryote, its rRNA gene is unlike any other eukaryote. In most eukaryotes, the rRNA gene units are identical in sequence and tandem arrayed in large numbers. Plasmodium species are encoded by a small number of rRNA gene units that are physically separated in the genome and variable in sequence (1, 2). Transcription studies indicate that eventually all of the genes are expressed over the course of the developmental cycle (3–6). Distinct types of mature 18 S rRNA have been shown to be associated with asexual parasites in the blood (A-type rRNA) (4, 5), gametocytes (this study), and developing sporozoites (S-type rRNA) (3, 7). Although different ribosomes perform the same basic function—translation of mRNA—functional variations exist between the types (8). Temperature is known to be one factor involved with transcriptional control in the developing sporozoite-stage rRNA (9), but regulation of the other rRNA copies is still elusive.

Glucose is related to rRNA transcription control in a number of other microbes (10, 11) and has been implicated in developmental transition in Plasmodium (12). There are dramatic drops in glucose concentration during transmission of the parasite from vertebrate to insect (13) as well as significant changes in glucose concentration in an infected human, especially within the microcirculation (14). One can anticipate that some of the changes in transcriptional patterns will parallel changes in glucose concentrations, even in culture. Some changes will be affected as a direct result of glucose starvation. Other changes involved in developmental transitions may well involve glucose but require additional factors including temperature change (9). Glucose concentration, the most important factor associated with the parasite growth, is addressed in this study as related to transcriptional control of rRNA.

**EXPERIMENTAL PROCEDURES**

*Parasite Culture and Separation Stages—*Plasmodium falciparum 3D7 was cultured according to standard methods (15). The parasites were synchronized twice with 5% sorbitol, and different asexual stages of parasites were collected. Gametocytes were induced and harvested as described (16). Oocysts and sporozoites of the parasite were obtained by feeding laboratory-reared *Anopheles gambia* mosquitoes with *P. falciparum*-infected red blood cells (17). The parasite used for glucose starvation is the unsynchronized mixed stages of parasite.

*Genomic DNA and RNA Isolation—*Genomic DNA from cultured lines of *P. falciparum* was isolated as described previously (18). Total RNA from the various parasite stages was isolated using Trizol (Invitrogen).

*Inverse PCR—*Inverse PCR was performed as described (19). The restriction enzymes HindIII and AflIII were used to clone fragments spanning the 18 S rRNA and promoters. The amplified PCR fragments were cloned into vector pCRII-TOPO (Invitrogen) and sequenced.

*Primer Extension and 5′-RACE—*Primers were used to map the transcriptional start sites of four rRNA genes as described (20). The RNA for A1 and A2 primer extension is from blood-stage parasites cultured at 37 °C. The RNA for S1 primer extension is from gametocytes. The RNA for S2 primer extension is from blood-stage parasites cultured at 26 °C for 3 h. Gametocytes were chosen for S1 initiation site determination due to the abundance of S1 type rRNA in gametocyte stage (see "Results"). The blood-stage parasites cultured at 26 °C were chosen for S2 initiation site determination due to high transcription level of S2 at this temperature (9). 5′-RACE (Invitrogen) was used as an alternative method to determine the transcriptional start site.

*Real-time PCR—*One microgram of DNase-treated RNA was converted to cDNA using a SuperScript first-strand synthesis system (Invitrogen). 5% of the cDNA was used for real-time PCR using a Light-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF503871, AF503870, AF503869, and AF503868.

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† The abbreviation used is: RACE, rapid amplification of cDNA ends.
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**Fig. 1.** Schematic map of inverse-PCR and characteristics of rRNA gene precursor regions. Regions between restriction enzyme sites indicate sequences obtained by inverse PCR. Arrows indicate the transcription start site of each rRNA gene. Wavy lines indicate 530-bp conserved regions preceding two A-type 18S rRNA; dots show locations of the 20-bp conserved region preceding all 18S rRNA. Numbers above arrows and below enzymes indicate the distance between the indicated sites and 5' end of mature 18S RNA.

**Fig. 2.** Characterization of promoters of A1, A2, S1, and S2 rRNA genes. A, primer extension of A1, A2, S1, and S2. +1 indicates the initiation site. B, sequences around initiation sites of rRNA gene of A1, A2, S1, and S2. Pink letters indicate initiation sites of each rRNA gene. Underlined letters indicate conserved regions and similar GC-rich regions between A1 and A2 rRNA genes. C, comparison of promoter structures of A-type rRNA genes in *P. falciparum* with promoter structure of rRNA gene in *E. coli*. Pink letters indicate conserved sequences between A-type rDNA promoter and *E. coli* rDNA promoter.
Cycle thermal cycler (Roche Applied Science) and detection of product by SYBR Green I. The primers for real-time PCR of four copies of rRNA are: A1, TTGGTATATGCAAAAATTAATATGTTT and TAATTCTATGAAAAGACATGGAACAC; A2, TTCGCTAATATTGGAAAATTCAG and CATTTGATGTTTTATTAATGTTATTATCTT; S1, CATCAATTGCGTTTGTTT and AACGATCAAACACACTAC; and S2, TAGCTAACAAATGACACTTTA and ATGAAAAACTTATACATATG.

Real-time PCR conditions for each gene was according to the Roche Applied Science protocol, except that the extension temperature was 60 °C (21). Specific cDNAs were quantified using a standard curve based on the known concentration of the plasmids that contained the same DNA fragment. The products were cloned into pCRII vector and sequenced. The sequences were as predicted.

RESULTS

Cloning rRNA Gene Precursor and Promoter Regions—It has been shown that both A-type rRNA and S-type rRNA genes in *P. falciparum* have multiple copies and different loci properties (18). The sequences preceding two mature A-type rRNA genes (A1 and A2, GenBank™ accession numbers AF503871 and AF503870, respectively), and two mature S-type rRNA genes (S1 and S2, GenBank™ accession numbers, AF503869 and AF503868, respectively) were identified by inverse PCR. A schematic of each precursor is shown in Fig. 1; transcriptional patterns of each rRNA gene (rDNA) are described in “Characterization of Precursors and Promoters of rRNA Genes.” The sequence data obtained here are the same as those in the recently released *Plasmodium* data base (PlasmoDB www.plasmodb.org).

Characterization of Precursors and Promoters of rRNA Genes—RNA from different blood-stage or low temperature-treated parasites was used as a template for mapping the initiation sites of different rRNA genes. The transcriptional start point for each of the four transcripts (Fig. 2A) was revealed by primer extension experiments and confirmed by 5'-RACE technique. Distances from the transcription starting points of each rRNA gene to the 5'-end of mature 18 S rRNA are: A1, 1145 bp; A2, 1042 bp; S1, 1184 bp; and S2, 1318 bp (Fig. 1). The full-length precursors of A1 and A2 rRNA contain a similar 530-nucleotide region preceding the mature 18 S rRNA, although the sequences upstream to this region are diverse. In contrast, the only homologous sequence relating S1 and S2 in the precursor is a 20-bp region immediately 5' to the mature 18 S rRNA sequence that is similar to the corresponding sequence in the A genes.

The region upstream to the initiation site is the putative rRNA gene promoter. No conserved sequences were found upstream to the S1 and S2 initiation sites. Surprisingly, the general structure of these A1 and A2 promoters is very similar.
to the rRNA gene promoter structure in *Escherichia coli* (22, 23). Conserved sequences encoding the putative promoter are seen in A1 and A2. Two conserved boxes similar to the −10 and −35 regions of *E. coli* rRNA gene promoter were identified upstream to the A1 and A2 transcription start sites. The distance between these two boxes is 61 bp in A1 and 64 bp in A2. Two GC-rich regions of 20 bp each, similar to that in the *E. coli* rRNA gene promoter, were identified upstream to the A1 and A2 transcription start site. The distances between the GC-rich regions and the second conserved box are 90 bp and 92 bp, respectively (Fig. 2, B and C). The fact that the *P. falciparum* genome is more than 80% AT makes these GC-rich boxes notable.

**Transcriptional Pattern of Different rRNA Genes**—Sequence analysis of the rRNA precursors revealed polymorphisms 5' to the mature 18S rRNA gene in all copies in “Characterization of Precursors and Promoters of rRNA Genes.” Their existence allowed us to design experiments to specifically detect transcripts from each of the four genes. To establish a baseline, RNA from various stages of parasite development was purified, and different copies of A- and S-type rRNA were quantified by detecting the transient precursor region by real-time PCR (Fig. 3). The results showed, as expected, that genes A1 and A2 were the predominant forms in asexual parasites. Full-length transcripts encoded by S1 are dominant in gametocytes but decrease in relative amounts as the parasite develops through the mosquito stages. S2 transcripts, first seen in overwhelming amounts in oocysts, are the dominant gene product in sporozoites.

**Glucose Concentration and rRNA Gene Transcription**—Transcriptional change as a result of glucose variation was measured at 37 °C. Aliquoted parasites were cultured at five glucose concentrations (2, 1, 0.5, 0.25, and 0.11 mg/ml) for 3 h. The normal glucose concentration in human sera ranges from 0.7 mg/ml to 1.3 mg/ml. 1 mg/ml represents the normal glucose concentration in human sera. Glucose concentration in severe malaria with hypoglycemia can be as low as 0.12 mg/ml (24) and even lower in a microcirculation system occluded by sequestrated parasites. We show that culture at the lowest glucose concentration (0.11 mg/ml) for 3 h does not affect parasite viability. Relative amounts of transcription from A1, A2, S1, and S2 genes were determined by real-time PCR (Fig. 4). Each experiment was performed on three independent occasions with different sample sources yielding consistent results. Transcription of A1 at 0.11 mg/ml glucose was decreased relative to the control (1 mg/ml) sample by more than 85% (Fig. 4A); transcription of A2 was decreased by 80% (Fig. 4B). No variation in transcriptional levels was observed for S1 (Fig. 4C). Transcription of S2 was found to increase as glucose level decreased (Fig. 4D).

The effect of low glucose on rRNA transcription was observed over a time course. Aliquoted parasites were separately cultured in control media containing 1 and 0.11 mg/ml glucose for 1, 2, and 3 h. Transcription of A1 and A2 decreased continually over time, whereas no changes were observed in S1. S2 transcription continued to increase as the starvation time increased (Fig. 5).

The viability of parasites under our starvation conditions was determined by recovery of A-type rRNA transcription. Blood-stage parasites were starved at 0.11 mg/ml for 3 h and then returned to 1 mg/ml glucose for another 3 h. Fig. 6 shows that the A gene transcription level fully recovered.

**A Synergistic Effect of Glucose and Temperature**—During

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**Fig. 5.** Time curve of the effect of glucose starvation on transcription levels of A1, A2, S1, and S2 rRNA genes in asexual blood-stage parasites.

**Fig. 6.** Recovery of transcription of A-type rRNA genes in parasites subjected to glucose deprivation. The copy number of the transcription of A1 and A2 rRNA genes at 1 mg/ml glucose concentration for 3 h was ranked as 1. The line graph shows fluorescence-cycle number from real-time PCR.
transmission of the parasite from the vertebrate host to the insect host, temperature change parallels glucose concentration change. We analyzed the influence of glucose concentrations and temperature change alone and in combinations. The results are shown in Fig. 7. Cultured parasites at 26°C for 3 h down-regulate the transcription level of A-type rRNAs (A1 and A2) close to 50%. Glucose starvation can down-regulate A1 and A2 transcription levels by 85 and 80%, respectively. The glucose starvation in combination with low temperature can further decrease the transcription of A1 to 90% and A2 to 84% (Fig. 7, A and B). The effect of the combination is too small to say anything about a cooperative effect. S1 is regulated differently than the other genes (Fig. 7C). Neither temperature nor glucose starvation have a significant effect the level of transcription.

The transcription level of S2 is affected by a combination of glucose starvation and low temperature in a synergistic manner. The glucose starvation alone can up-regulate S2 transcription by 50% whereas low temperature alone can increase S2 transcription 16-fold. Glucose starvation in combination with low temperature has a synergistic effect increasing S2 transcription by 49-fold (Fig. 7D) in what appears to be a collaborative association.

DISCUSSION

Developmental transitions in eukaryotic microorganisms relating to glucose starvation and low temperature in a synergistic manner. The glucose starvation alone can up-regulate S2 transcription by 50% whereas low temperature alone can increase S2 transcription 16-fold. Glucose starvation in combination with low temperature has a synergistic effect increasing S2 transcription by 49-fold (Fig. 7D) in what appears to be a collaborative association.

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FIG. 7. The synergistic effect of glucose starvation and low temperature on the transcription of A1, A2, S1, and S2 rRNA genes. Copy number of the transcription of each rRNA gene at 1 mg/ml glucose concentration and 37°C was ranked as 1. The line graph shows the fluorescence-cycle number from real-time PCR. A–D, relative transcription levels of A1 (A), A2 (B), S1 (C), and S2 (D) rRNA at different temperature and under different glucose concentrations in blood-stage parasites.
or opened. The ratio of activating and inactivating rRNA genes is tissue-specific (30). It has been shown that silencing of rRNA genes is often methylation-dependent (31). We tried to determine if the regulation of rRNA genes in *P. falciparum* is methylation-dependent by using methytransferase inhibitor, 5-azacytidine, which can inhibit the inactivation of rRNA genes in mouse cell. We were unable to detect a relationship between methylation and transcriptional regulation of rRNA genes in the parasite.

There are a number of parallels with the type of control described here for *P. falciparum* with that initially described for *E. coli* and is referred to as the “stringent response.” Carbon source starvation or amino acid starvation in *E. coli* can cause the dramatic inhibition of the synthesis of rRNA, tRNA, and mRNA for ribosomal proteins as well as selective pattern changes in other mRNAs (10). The definition of a stringent response has been broadened to include organisms such as yeast (11), which also selectively reduce the transcription of ribosomal RNA and mRNAs of ribosomal protein in response to external signals such as amino acid starvation. Interestingly, it is now known that the stringent response in prokaryotes is dependent on the GC-rich region just preceding the initiation site of transcription (32–34). The similar GC-rich sequences in the promoters of A-type rRNA genes in *P. falciparum* suggest that the reciprocal regulation of different types of ribosome RNA genes in *P. falciparum* under glucose starvation fits into this category. Identifying the specific proteins or enzymes involved would be beneficial to the development of new drug target.

Acknowledgments—We thank David Keister for the mosquito infection experiment and Nancy Shulman for editorial advice and support.

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