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Transcriptional analysis of genes encoding enzymes of the folate pathway in the human malaria parasite *Plasmodium falciparum*

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Summary

Folate metabolism in *Plasmodium falciparum* is essential for cell growth and replication, and the target of important antimalarial agents. The pathway comprises a series of enzymes that convert GTP to derivatives of tetrahydrofolate, which are cofactors in one-carbon transfer reactions. We investigated the expression of five of the genes encoding these enzymes by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using a threshold detection technique. We followed changes in mRNA levels as parasites progress through the erythrocytic cell cycle and examined this process in two cloned lines of diverse origins, as well as under stress conditions, induced by either removal of important metabolites or challenge by folate enzyme inhibitors. Although conventionally regarded as performing housekeeping functions, these genes show disparate levels of and changes in expression through the cell cycle, but respond quite uniformly to folate pathway-specific stress factors, with no evidence of feedback at the transcriptional level. Overall, the two genes involved in the thymidylate cycle (encoding dihydrofolate reductase–thymidylate synthase, *dhfr-ts*, and serine hydroxymethyltransferase, *shmt*) gave the most abundant transcripts. However, only the latter showed major variation across the cell cycle, with a peak around the time of onset of DNA replication, possibly indicative of a regulatory function.

Introduction

Antifolate inhibitors are of major clinical utility in combating the lethal species of the protozoan malaria parasite of humans, *Plasmodium falciparum*. These drugs represent an important line of defence against the widespread strains of chloroquine-resistant *P. falciparum*, targeting two enzymes, dihydropteroate synthetase (DHPS) and dihydrofolate reductase (DHFR) in the folate pathway of the parasite (Fig. 1). Inhibition of these activities disrupts the constant supply of tetrahydrofolate (THF) cofactors needed for critical one-carbon (1-C) transfer reactions, including that for DNA synthesis. Cloning of the *pppk-dhps* (Brooks *et al*., 1994; Triglia and Cowman, 1994) and *dhfr-ts* (Bzik *et al*., 1987) bifunctional genes encoding these activities was essential for understanding the genetic basis of antifolate drug resistance. Recently, genes encoding other enzymes in the folate pathway, which represent potential new targets for chemotherapeutic intervention, have been cloned and characterized (Alfadhli and Rathod, 2000; Lee *et al*., 2001; Salcedo *et al*., 2001). Thus, of the genes encoding the nine enzyme activities depicted in Fig. 1, which lead to fully reduced polyglutamated folate derivatives, only that for dihydroneopterin aldolase (DHNA) has remained elusive, despite near completion of the parasite genome sequence. The identification of almost all the genes of the pathway permits an inclusive experimental approach to their expression for the first time.

The pathway may be conveniently divided into two sections: those enzymes involved in the biosynthesis of the folate moiety from the simple precursors GTP, *p*-aminobenzoic acid (pABA) and glutamate (GTPCH, DHNA, PPPK, DHPS and DHFS; for abbreviations, see Fig. 1) and those that interconvert the folate among its various forms used in one-carbon transfer reactions (DHFR, TS, SHMT and FPGS). *P. falciparum* is able to salvage preformed folate as well as synthesize it *de novo* (Krungkrai *et al*., 1989). However, the precise mechanism(s) by which this occurs is not known, nor is the degree to which the parasite can alter the balance between synthesis and salvage, depending upon its external environment, although there is evidence that this may vary among strains (Wang *et al*., 1997a). To understand better the function(s) of each of the genes in the pathway, information is required about the dynamics of their expression, i.e. whether there are changes during normal progression through the parasite life cycle or in response to

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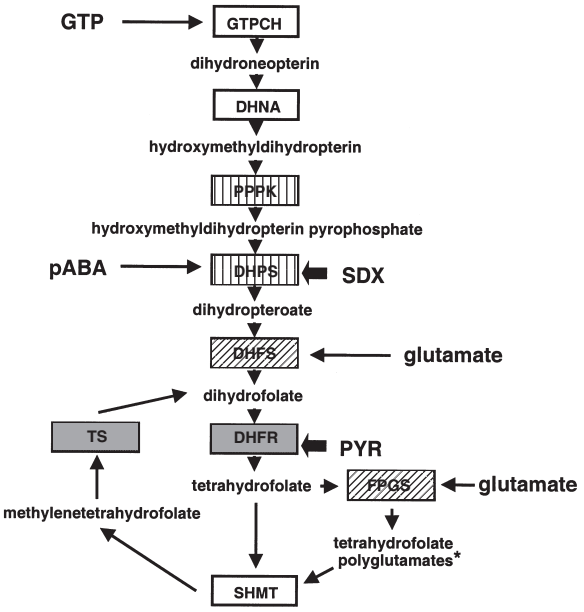

Fig. 1. The folate pathway showing the functions of the enzymes encoded by the genes analysed in this work. GTPCH, GTP cyclohydrolase I; DHNA, dihydroneopterin aldolase; PPPK, hydroxymethyldihydropterin pyrophosphokinase; DHPS, dihydropteroate synthase; DHFS, dihydrofolate synthase; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthase; SHMT, serine hydroxymethyltransferase; TS thymidylate synthase. Only the *dhna* gene remains to be identified in *Plasmodium*. DHPS and DHFR are targeted in current antimalarial chemotherapy by drugs such as SDX and PYR respectively. Boxes shaded with the same pattern indicate activities encoded by bifunctional genes. The asterisk indicates that tetrahydrofolate with differing levels of glutamation may be passed through the thymidylate cycle.

varying external influences on the parasite. Such information will be an important element in any future rational design of novel antifolate inhibitors.

Although regulation of mRNA level is not necessarily the sole determinant of protein abundance, and correspondence between mRNA and protein expression levels is often poor or lacking, differences in cell type or state can usually be correlated with changes in the mRNA levels of the genes involved (DeRisi *et al*., 1997). Ideally, both protein and mRNA gene expression patterns would be determined for every gene (Hatzimanikatis and Lee, 1999), especially as mRNA transcripts in *P. falciparum* have in general very long 5′ untranslated regions (5′ UTR) (Watanabe *et al*., 2002), which might play a role in translational regulation. However, it is not yet possible to identify or quantify all the malarial folate pathway enzymes on one- or two-dimensional gel systems, as they are very-low-abundance proteins, and a complete set of the corresponding antibodies is not yet available.

Here, we describe a quantitative reverse transcription-polymerase chain reaction (qRT-PCR) procedure that permits comparisons among all the characterized genes of the *P. falciparum* folate pathway at the level of transcription. We used this to examine changes in expression of these genes as the parasite proceeded through its life cycle in human erythrocytes, and as external factors influencing folate metabolism were varied. We wished to address several important questions: (i) are these genes, usually classified as 'housekeeping', constitutively expressed?; (ii) if not, how does the relative abundance of their transcripts vary across the cell cycle, particularly in relation to the timing of DNA synthesis?; (iii) do significant differences occur between strains of the parasite known to differ in their ability to take up or use preformed folate?; and (iv) how does the transcription of these genes respond to external stress, in the form of either depletion of folate and its precursor pABA or challenge by the clinically important antifolate drug combination of pyrimethamine (PYR) and sulphadoxine (SDX)? We found that there are marked variations in the levels of the different folate pathway transcripts as the cell cycle progresses, particularly that of *shmt*, but that responses to either drug or metabolite stress are fairly uniform across the gene repertoire. Although antifolate drugs are in widespread use against a range of both bacterial and eukaryotic pathogens, to our knowledge, this study represents the first multigene analysis of transcriptional regulation focused on the folate pathway in any organism.

Results

Calibration and verification of the qRT-PCR system

Although many published reports purport to quantify mRNA using end-point data from RT-PCRs, measurements are more reliable when derived from the start of the exponential phase of amplification where product is first detectable. As the reaction proceeds, PCR efficiency is increasingly affected by limiting reagents, exact cycling conditions and small differences in reaction components, such that, at the plateau (end-point) phase, quantification can be severely compromised (Higuchi *et al*., 1993; Wittwer *et al*., 1997). The principle of threshold PCR for measuring the abundance of a given sequence relies on an inverse linear relationship between the cycle number at which the amplification product is first detected above the baseline level (the threshold cycle; Ct) and the logarithm of the amount of genomic DNA (gDNA) or complementary DNA (cDNA) (Higuchi *et al*., 1993). We designed primer pairs (Table 1) to yield PCR products of comparable sizes for each of the *P. falciparum* folate pathway genes and, where possible (for *pppk-dhps*, *dhfs-fpgs* and *shmt*; Brooks *et al*., 1994; Triglia and Cowman, 1994; Lee *et al*., 2001), to span introns to permit monitoring of contaminating gDNA in the RNA sample before and after DNase treatment. We used gel electrophoresis to monitor

Table 1. Oligonucleotide primers used for RT-PCRs.

Gene	Accession number[a]	Forward primer (RNA synonymous)	Reverse primer	Annealing temperature	Fragment sizes (bp)[b]
gtpch	AF043557	GAATGATAATAAAAGGTTAGGTAGC	TTGTAATCTTCTAGAAAAGACATC	57°C	646
pppk	Z31584	CTAGAAACTGCTCTGCACCTTG	TTGTTCTTTCATCCTACTCA	54°C	990
					1182
dhps	Z31584	GGAATACCTCGTTATAGG	TTGTAGAATTCACTTGGTCTATTTTTG	54°C	433
					553
dhfs-fpgs	AF161264	CACAATGAAACGGCAATAGATAG	GGTTCATTCATAAAAATGGTATCTTGC	57°C	500
					920
dhfr[c]	M22159	TCTTCTAGATATGATGGAACAAGTCTGCG	TGTGAATTCAACATTTTATTATTCGTTTTC	54°C	720
ts	M22159	AAGTGATCGAACGGGAGTAGGTG	TGTTGGGAATGGATAGGGTATTC	54°C	694
shmt	AF195023	TGCAAAAATATGACAAGGAGCTC	CAACTATAAGATGATTATCGGTTC	54°C	969
					1128
ssu	M19172	CATTCGTATTCAGATGTCAGAGGTG	CGTTCGTTATCGGAATTAACCAGAC	50°C	482
ssuRT[d]	M19172		GCTTACTAGGCATTCCTCGTTGAAG	42°C	

a. Complete gene sequences in the GenBank-EMBL database used to design primers; *pppk* and *dhps* constitute two distinct domains of a bifunctional gene (Brooks *et al*., 1994; Triglia and Cowman, 1994), as do *dhfr* and *ts* (Bzik *et al*., 1987), whereas *dhfs* and *fpgs* are co-activities on a single domain (Salcedo *et al*., 2001) (see also Fig. 1).
b. Where two sizes are given, the larger includes the intron(s) found in genomic DNA within the compass of the primers.
c. These primers were originally designed for expression purposes and thus carry restriction sites (underlined) that do not match the genomic sequence.
d. Primer used to initiate first-strand cDNA synthesis from 18S (SSU) rRNA.

the PCR, as the intensity of the desired product of known length can be followed specifically, unlike with some automated systems, in which there is no discrimination between fluorescence from the target sequence and from non-specific products such as primer dimers, unless complex melting experiments are also conducted in parallel on a continuous monitoring facility (Morrison *et al*., 1998).

The system was initially calibrated using genomic DNA of known concentration. As all the folate pathway genes involved appear to be single copy (Snewin *et al*., 1989; Brooks *et al*., 1994; Lee *et al*., 2001), Ct values for each would be expected to be closely similar if the primers chosen permitted amplification with roughly equal efficiency. This was found to be the case, with Ct values all falling at cycle 21–22 in reactions containing 20 ng ml^{-1} DNA (Figs 2A and B). The calibration curves were linear over at least a three-log range of DNA concentration (500 pg ml^{-1}–500 ng ml^{-1}) with near-identical gradients for all the genes, thus validating the approach. These calibration curves were then used to quantify the absolute amount of cDNA for each of the genes produced after

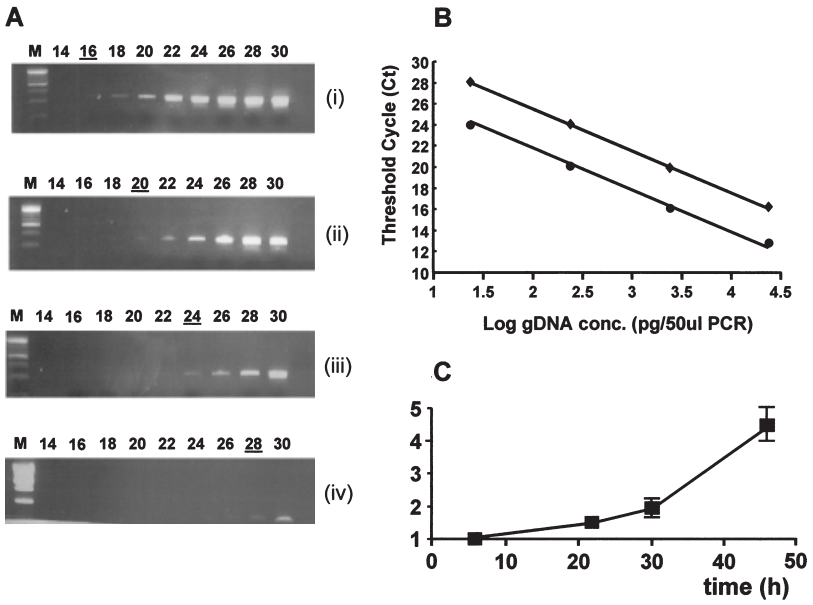

Fig. 2. Calibration of quantitative PCRs.
A. *shmt* amplified from a serial 10-fold dilution of 0.5 mg ml^{-1} stock K1 genomic DNA template: (i) 24 ng; (ii) 2.4 ng; (iii) 0.24 ng; (iv) 0.024 ng per 50 µl PCR. Lane numbers correspond to the cycle at which sampling was done, with threshold cycles (Ct) underlined.
B. Log_{10} of genomic DNA concentration in pg per 50 µl PCR was plotted against the corresponding threshold cycle to derive the standard curves for *shmt* (upper line) and *ssu* (18S rDNA; lower line). Curves derived for the other folate pathway genes were nearly superimposable on that for *shmt* and are omitted for clarity. That for 18S rDNA has an identical gradient but is displaced downwards because of the multiple copies present in the genome (Langsley *et al*., 1983).
C. Increase in 18S rRNA transcript level across the 48 h asexual cycle relative to the early ring stage (6 h time point).

reverse transcription of mRNA preparations from the various parasite samples. As in any cDNA-based quantification method, including microarray, expressed sequence tag (EST) or serial analysis of gene expression (SAGE) assays, the assumption is implicit that reverse transcription of each of the different mRNAs in the population is equally efficient.

A major consideration in any quantitative technique is how to normalize the data across different samples and experiments. In other systems, mRNAs encoding abundant proteins such as actin, tubulin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been used extensively as internal standards, on the assumption that they are constitutively expressed. However, this has been shown to be generally fallacious (Goidin *et al*., 2001). In the case of *P. falciparum*, marked variation of actin and tubulin expression across the cell cycle has also been observed (Wesseling *et al*., 1989; Delves *et al*., 1990; Calvo *et al*., 2002), and a recent microarray study (which did not include the folate pathway genes) confirmed that levels of both actin and GAPDH mRNA fluctuated by up to 10- and 48-fold respectively (Ben Mamoun *et al*., 2001). We therefore favoured 18S rRNA as a better internal standard, as (i) rRNA is present in much larger quantities than any mRNA species (rRNA constitutes >80% of the total RNA in *P. falciparum*, total mRNA ≈ 5%; Hyde *et al*., 1984) and therefore provides a denominator in the normalization ratio that is much less prone to experimental error at all stages of the cell cycle than another mRNA species would be; (ii) levels of rRNA should be largely unaffected by changes in RNA polymerase II transcription; and (iii) such levels have been shown to be constant in other systems (Goidin *et al*., 2001). The calibration curve using genomic DNA for this sequence was also linear over 3 logs and required less DNA to achieve the same threshold cycle as the folate genes (Fig. 2B), consistent with the multicopy nature of the 18S rDNA genes (Langsley *et al*., 1983). When we measured the absolute level of 18S rRNA transcripts across the *P. falciparum* asexual cell cycle for two different parasite lines, we observed a moderate but smooth increase of about four- to fivefold from ring to schizont (Fig. 2C). Levels of each folate pathway transcript calculated from their individual calibration curves were therefore normalized to that of 18S rRNA in the same sample for comparative purposes.

For most experiments, we also set up independent RT-PCRs based on both *dhfr* and *ts* sequences of the bifunctional *dhfr-ts* gene and on the *pppk* and *dhps* sequences of the bifunctional *pppk-dhps* gene. These measurements gave a good internal control of the processivity of the reverse transcriptase as well as of general reproducibility, as the levels measured, coming from two different domains of the same transcript, should be identical if the polymerase is proceeding along the complete length of the mRNA molecules. We found that this was always the case for *dhfr* and *ts* (>70 measurements of each; see for example Fig. 3A and B). However, small differences were occasionally seen between *pppk* and *dhps*, although neither domain was favoured, consistent with our observation (see below) that the *pppk-dhps* gene is always expressed at a much lower level than *dhfr-ts* and hence is more prone to random experimental error.

Changes in expression of the folate pathway genes through the blood stages of P. falciparum

Initially, we examined the levels of mRNA for each gene in asynchronous cultures of three different parasite strains of disparate origins, K1 (Thailand), HB3 (Honduras) and Dd2 (Indo-China). Although each strain gave generally similar patterns, it was clear that certain transcripts were quite variable, depending upon the distribution of rings, trophozoite and schizont stages in the mixed culture (data not shown). As this was indicative of changes in mRNA

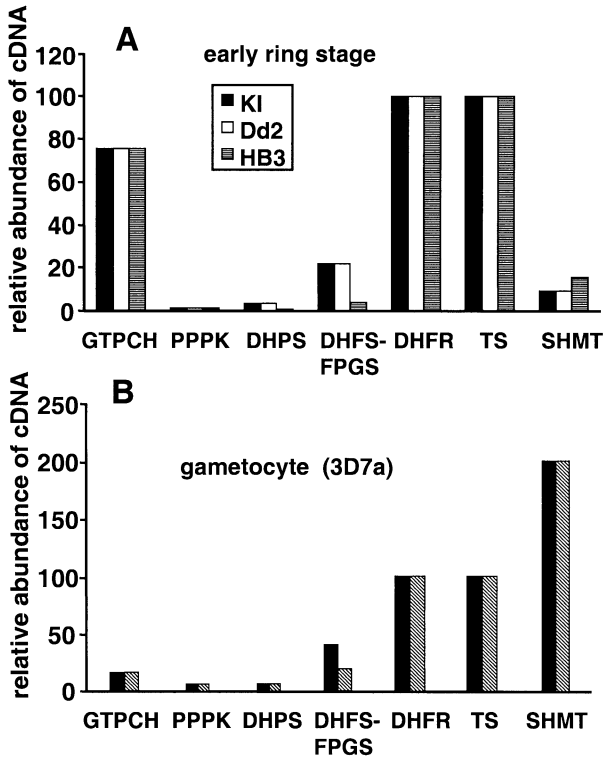

Fig. 3. Comparative expression of folate pathway genes in (A) ring (8 h) stage K1, Dd2 and HB3 strains of *P. falciparum* and (B) gametocyte (presexual) stages of the life cycle, as assayed in late-stage 3D7a gametocytes (two independent determinations). In both (A) and (B), levels of the *dhfr* and *ts* domains of *dhfr-ts* and of the *pppk* and *dhps* domains of *pppk-dhps* were measured independently to gauge reproducibility. The *dhfr* and *ts* transcripts, found to be identical, were normalized to a relative abundance of 100.

levels across the cell cycle, we then synchronized K1, HB3 and Dd2 cultures twice before sampling and compared the early (8 ± 2 h ring) stages of erythrocytic infection, while synchrony was still tight. After setting the level of the most abundant transcript (*dhfr-ts*) in each case to an arbitrary value of 100, a very similar pattern was seen for all these strains (Fig. 3A); notably, the level of the *pppk-dhps* gene transcript was very low (≈ 50× lower than that for *dhfr-ts*), whereas those of *gtpch*, *dhfs-fpgs* and *shmt* were intermediate between these extremes.

For subsequent experiments, we concentrated on comparisons between just HB3 and Dd2 for three reasons: (i) these cloned lines are the parents of one of the two genetic crosses carried out in *P. falciparum* (Wellems *et al*., 1990; Walker-Jonah *et al*., 1992); (ii) they have quite different *dhps* and *dhfr* genotypes and hence susceptibilities to antifolate drugs (Wang *et al*., 1997a); and (iii) they show important differences in their apparent ability to use exogenous folate (Wang *et al*., 1997a). Transcript levels were measured at intervals throughout the 48 h erythrocytic cycle for each gene. The gene that consistently gave the most stable pattern of transcription relative to that of 18S rRNA was *dhfr-ts*, with almost constant normalized levels over the cell cycle. In contrast, the most striking alteration in the early ring-stage pattern described above was found in the relative level of the *shmt* transcript, which rose steeply through the cycle in both Dd2 and HB3 from a very low level in young rings, plateauing at the late trophozoite/early schizont stages (Fig. 4A), at which point it was the most abundant of all the transcripts. In Fig. 4B, the kinetics of this increase is compared with that of DNA as replication to the multinucleate schizont occurs (Smeijsters *et al*., 1994). Significantly, the *shmt* transcript reaches its maximum relative level just as S-phase begins at ≈ 30 h into the cycle and stays high throughout the period of DNA synthesis. The *shmt* transcript also represented the most abundant species in gametocyte mRNA isolated from 3D7a (Fig. 3B), which is a cloned line competent in producing high levels of infective gametocytes in culture (Walliker *et al*., 1987). The other transcript that showed a significant cyclical change was that for *dhfs-fpgs*, which also rose through the trophozoite stage to a maximum but, unlike the *shmt* transcript, diminished at the schizont stage and was also quite low in gametocytes (Figs 3B and 5). The *gtpch* transcript levels appeared to be more prone to experimental variation among similar cultures measured at different periods, although a peak at the late trophozoite stage was normally observed, with a very low level in gametocytes. Overall, the genes encoding the enzymes of the thymidylate cycle (*dhfr-ts* and *shmt*) appear to be expressed at the highest levels, with that for *pppk-dhps* at the lowest, at all stages of the erythrocytic cycle.

Heterogeneity in the 5′ UTR of dhfr-ts *and transcription*

As differences in the apparent folate salvage capabilities of HB3 and Dd2 have been broadly mapped to the *dhfr-ts* locus (Wang *et al*., 1997a), we examined the promoter region of this gene in the two clones, as a possible source of heterogeneity. PCR mapping across the entire upstream intergenic region (i.e. as far as the next open reading frame) revealed a major difference between the two clones close to the start of transcription, which we found by DNA sequencing to result from the presence of two tandemly repeated units of a 256 bp sequence in Dd2 just downstream of the major transcription start point (*c.* −790 in Dd2), which is present in only one copy in HB3 (Fig. 6A and B). It was important to assay whether this difference in the mRNA-encoding sequence influenced the absolute levels of the *dhfr-ts* transcript when compared between the two clones. However, the ratio of this transcript to that of 18S rRNA was found within experimental error to be the same in each case (Fig. 6C), indicating that, if this marked sequence difference within the

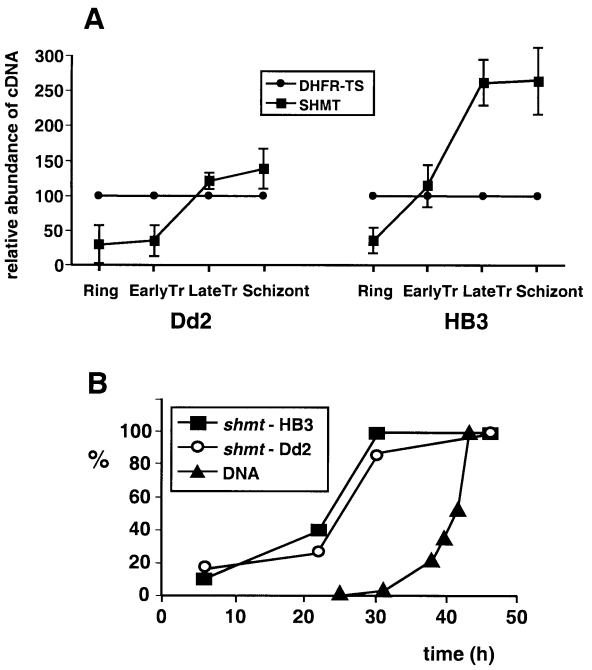

Fig. 4. A. Comparative expression of the genes involved in the thymidylate cycle over the asexual life cycle, divided into ring (4–8 h), early trophozoite (20–24 h), late trophozoite (28–32 h) and schizont (44–48 h) stages, for clones Dd2 and HB3. The levels of the *dhfr-ts* transcript stayed constant relative to 18S rRNA and were set at a relative abundance of 100. The levels of *shmt* are mean values ($n = 4$) ± standard deviation.
B. Expression of the *shmt* transcript in Dd2 and HB3 over the 48 h asexual cycle compared with the average increase in DNA content per parasite. Mean levels of *shmt* taken from (A) are expressed relative to the peak normalized values (100%) in each case. Data for DNA multiplication were taken from Smeijsters *et al.* (1994).

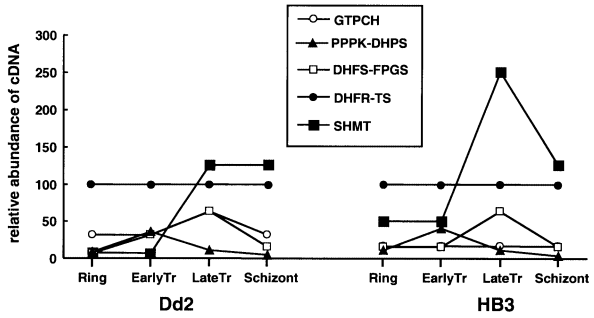

Fig. 5. Comparative expression of the folate pathway genes over the asexual life cycle, divided into ring (6–10 h), early trophozoite (18–22 h), late trophozoite (30–34 h) and schizont (42–46 h) stages, for clones Dd2 and HB3. The fall in the *shmt* level in this HB3 set at the end of the cycle resulted from some dilution of the schizonts with newly formed ring stages expressing very little *shmt*. The levels of the *dhfr-ts* transcript stayed constant relative to 18S rRNA and were set at a relative abundance of 100. Error bars, of similar magnitude to those in Fig. 4A, are omitted for clarity.

5′ UTR has any significant effect on expression, it must be at the post-transcriptional stage. Although the levels of the *dhfr-ts* transcript were the same, we noticed a consistent difference in the peak levels of the *shmt* transcript between HB3 and Dd2 (Fig. 4A). Over four experiments on synchronized cultures, the mean peak ratio of *shmt* to *dhfr-ts* was 1.4 ± 0.4 (standard deviation) in Dd2 and 2.6 ± 0.5 in HB3.

Comparison of transcript levels in parasites subject to folate deprivation

Certain strains of *P. falciparum*, such as Dd2, appear to be able to make efficient use of exogenously supplied folate and thus largely bypass inhibition of DHPS by sulpha drugs when administered alone, whereas others, such as HB3, appear to be much less efficient in exploiting folate salvage (Wang *et al.*, 1997a). To see whether deprivation of exogenous folate would induce an upregulation of those genes involved in *de novo* synthesis or otherwise affect the expression of any of the folate pathway genes in these two lines, cultures of each clone were split. One half was perpetuated in standard RPMI 1640, which has a high concentration of both folate and one of its precursors, pABA (1 μg ml^{-1} each), and the other in the same medium devoid of folate and pABA. Initial experiments were conducted on parallel asynchronous cultures, which were sampled over periods of up to 12 days. For both lines, no differences were seen in expression patterns in the first 2 days. After that time, only slight differences were observed up to the point at which cultures in the depleted medium were becoming sufficiently unhealthy to preclude the reliable extraction of mRNA samples.

To examine whether any differential effects were being masked by the use of asynchronous cultures, similar experiments were conducted on parallel cultures of synchronized HB3 and Dd2 parasites. Again, no major differences were seen between the parasite extracts from complete and depleted media over a period of 4 days (Fig. 7). Occasionally, strain variation was seen in the levels of *shmt* and *dhfs-fpgs*, but these were not reproducible and not consistent as the time of depletion increased. They therefore probably resulted from small differences in the synchrony between the complete and depleted medium cultures as the experiments progressed, given the variability of these gene transcripts across the cell cycle (Fig. 5).

Comparison of transcript levels in parasites subject to antifolate drug challenge

To simulate conditions *in vivo* in which antifolate drugs are most often administered together as Fansidar (per tablet: SDX, 500 mg; PYR, 25 mg), parallel cultures of HB3 and Dd2 were treated with both these drugs in a weight ratio of SDX:PYR of 50:1. This was chosen to mimic the peak plasma ratios measured in patients (Watkins *et al.*, 1997).

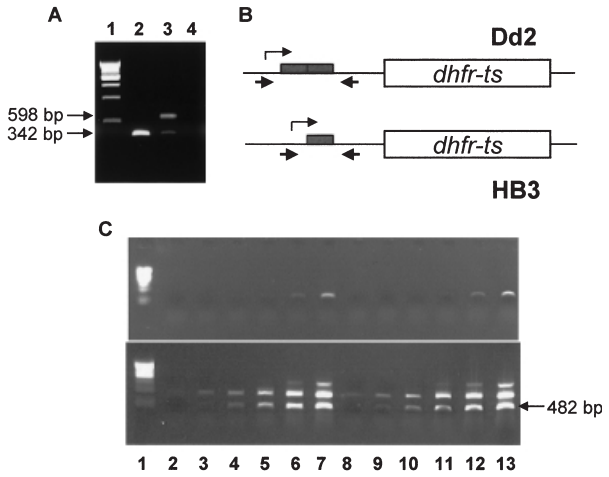

Fig. 6. Heterogeneity in the 5′ UTR of the *dhfr-ts* genes of HB3 and Dd2.
A. PCR products from primers flanking the repeat region: lane 1, size markers; lane 2, HB3 genomic DNA template; lane 3, Dd2 genomic DNA template; lane 4, negative control.
B. Schematic interpretation of the PCR and sequencing results. The longer arrows indicate the start of transcription (Chan, 1991) and the shaded boxes the tandem 256 bp repeat units; the shorter arrows represent the positions of the diagnostic PCR primers.
C. Expression of the *dhfr-ts* transcript of HB3 and Dd2 relative to 18S rRNA: lane 1, size markers, lanes 2–7, HB3; lanes 8–13, Dd2. Upper gel, PCR products from successive cycles using *dhfr* primers; lower gel, using *ssu* primers. The multiple bands in the latter are a consequence of heterogeneity in the organization of the several copies of the rDNA genes (Langsley *et al.*, 1983); the threshold cycle was taken as that at which the smallest (482 bp) product appeared (see Table 1).

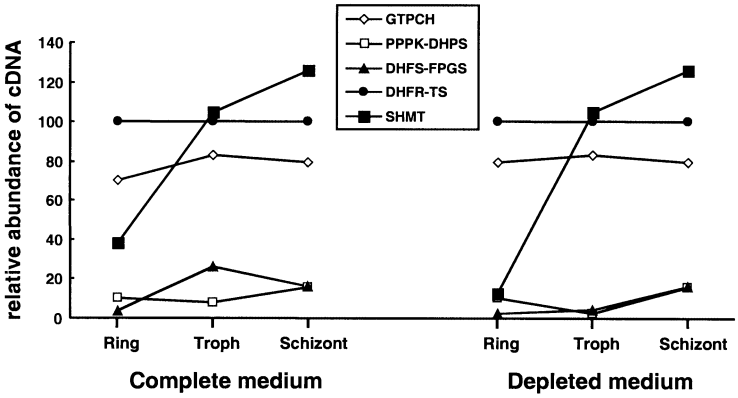

Fig. 7. Comparative expression of the folate pathway genes in synchronized Dd2 parasites grown in folate-replete and folate-depleted medium. Parasites passed through a complete cycle before sampling. The levels of the *dhfr-ts* transcript stayed constant relative to 18S rRNA and were set at a relative abundance of 100. Error bars, of similar magnitude to those in Fig. 4A, are omitted for clarity. Similar data were obtained for HB3.

As these two clones have different genotypes with respect to antifolate drug resistance (HB3 is wild type in *dhps*, singly mutant in *dhfr*, whereas Dd2 is triply mutant in both genes; Wang *et al*., 1997a), it was necessary first to titrate drug levels to have comparable effects on the two cultures. This was done by assessing at what level similar morphological and growth changes had taken place by the time of the trophozoite stages of the cycle following drug administration, as monitored by both microscopy and [^{3}H]-hypoxanthine incorporation. As expected, the concentration of the drug required to observe these effects was much higher (5×) in the case of Dd2. The appropriate level of drug was then added mid-cycle to synchronized cultures of HB3 and Dd2, and samples were removed for mRNA extraction and qRT-PCR at 26 h after administration, during the late schizont period of the same cycle, then at the ring, early trophozoite and late trophozoite stages of the following cycle, by which time the appearance of the parasites was clearly abnormal, and beyond which no further division was observed. Up to the ring stage, no differences were seen for any of the gene transcripts between drug-treated and control cultures. From the early trophozoite to late trophozoite period, however, the transcript levels of all the genes were significantly reduced, to about 10–40% of levels in controls, for both HB3 and Dd2 (Fig. 8A). This is clearly illustrated by the difference plots in Fig. 8B, where all values for untreated (control) parasites are set at 100 across the cycle. This compared with a reduction in the level of RNA polymerase I transcription, producing the 18S rRNA, to only about 70% of the control level.

Discussion

Folate metabolism is of crucial importance to all living organisms and has long been the target of antibacterial

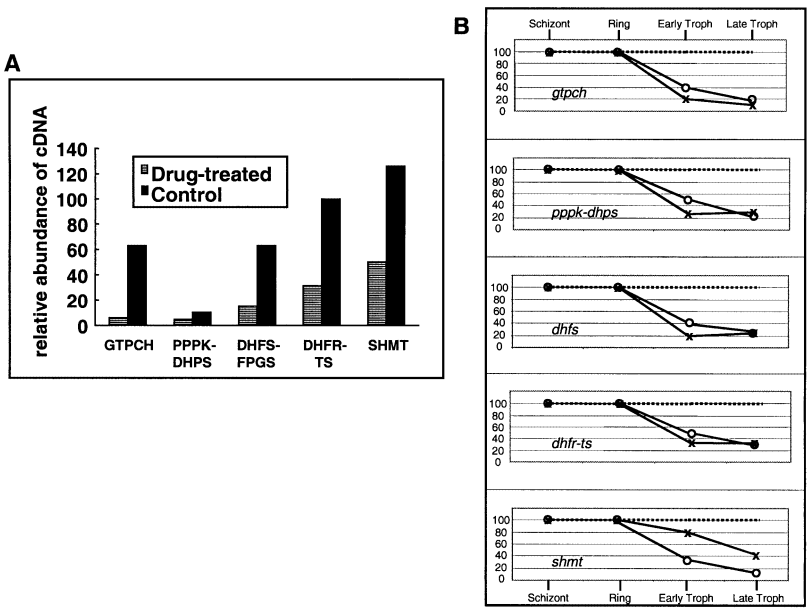

Fig. 8. Comparative expression of the folate pathway genes in the presence and absence of PYR-SDX added mid-cycle.
A. Transcription in Dd2 at the late trophozoite stage of the following cycle.
B. Difference plots for each gene transcript showing the level of suppression at each stage of the life cycle after drug addition for Dd2 (crosses) and HB3 (circles), relative to untreated controls (dotted lines), all set to an abundance of 100.

and antiprotozoal chemotherapy. To build up a more complete picture of the folate pathway in malaria parasites, we have monitored the levels of expression for genes encoding the enzymes of *P. falciparum* as it progresses through its cell cycle under different conditions. To calibrate, control and compare these measurements among the family of genes in a meaningful manner, we used six procedures: direct observation of the specific RT-PCR product; external standard curves based on genomic DNA for each primer pair; internal controls exploiting bifunctional genes; primer design to encompass introns; DNase treatment to eliminate interference from residual genomic DNA; and normalization to the level of 18S rRNA in each sample, which increases only moderately across the life cycle and the abundance of which relative to all mRNA species provides a robust divisor in the calculation of ratios.

With the possible exception of *dhfr-ts*, which tracks the level of 18S rRNA closely, we found that the folate pathway genes in *P. falciparum* cannot be regarded as 'housekeeping' in the sense of displaying constitutive expression levels across the cell cycle. Unlike a typical mammalian cell, an individual parasite is undergoing considerable changes in morphology, metabolism and nuclear content as it proceeds through the 48 h erythrocytic cycle. The major alteration in pattern that we observed was the dramatic increase in levels of *shmt* mRNA as the parasites developed from the ring stage through to mature schizonts. This was reproducible in three different parasite lines and was observed whether or not the parasites were provided with an exogenous source of folate. Although the gametocyte mRNA that we analysed was not derived from the lines used for the bulk of the experiments, the level of *shmt*, the most highly represented message in late trophozoites and schizonts, was also the highest of the set in this presexual stage.

SHMT catalyses a reversible reaction in which the side-chain of serine is transferred as a 1-C unit onto THF to yield 5,10-methylene THF, which in turn is used by TS to methylate dUMP, giving the dTMP needed for DNA synthesis. During the latter reaction, the THF moiety is oxidized to the dihydrofolate (DHF) level and must be reduced back to THF by DHFR, completing the so-called thymidylate cycle. Of the reactions involving folate cofactors, the formation of thymidylate is the most critical. Thus, malaria parasites salvage purines, rather than use folate-dependent synthesis (Sherman, 1979), and requirements for methionine can apparently be satisfied without using the side-chain of serine via 5,10-methylene THF and methyl THF (Asawamahasakda and Yuthavong, 1993). However, as pyrimidine salvage is not used (Sherman, 1979), the parasites are entirely dependent on the thymidylate cycle for replication. This may explain why levels of the *dhfr-ts* and *shmt* transcripts involved are the highest once the parasites move out of the ring stage. All folate required for replication, whether synthesized *de novo* or salvaged, must be converted to 5,10-methylene THF by SHMT and the DHF resulting from dTMP formation reduced back stoichiometrically to THF by DHFR. However, although the level of *dhfr-ts* remains quite constant relative to 18S rRNA across the cell cycle, that of *shmt* changes dramatically, indicating a possible regulatory function for the expression of this gene. SHMT can be regarded as the first enzyme in the thymidylate cycle, in that it is directly responsible for the introduction of a 1-C unit into the folate-processing pathways. The supply of serine itself is unlikely to be limiting, as this is not an essential amino acid in culture (Divo *et al*., 1985) and is therefore presumably derived in sufficient quantities by breakdown of haemoglobin, uptake from host plasma or via glycolysis, which proceeds at very high rates in blood-stage parasites. Experiments in *Saccharomyces cerevisiae* (Pasternack *et al*., 1994) and mammalian cells (Oppenheim *et al*., 2001) indicate that the TS activity is sufficient to process methylene-THF immediately as it is formed, with the rate of entry of 1-C units into the folate pathway regulated by cytoplasmic SHMT. Thus, in fast-dividing systems such as cancer cells, levels of SHMT are elevated to permit the high degree of incorporation of 1-C units required to sustain the quantities of dTMP needed for rapid doublings of the DNA content (Snell *et al*., 1987; Oppenheim *et al*., 2001). Analogously, rapidly proliferating malaria parasites also have a high demand for DNA components, with an average increase in DNA content per parasite per 48 h cycle measured at ≈ 14-fold, when development to the multinucleate schizont is complete (Smeijsters *et al*., 1994). Moreover, it is well documented that the onset of DNA synthesis occurs at the late trophozoite/early schizont stage ≈ 28–32 h into the cycle and is complete by about 40–44 h (Inselburg and Banyal, 1984; Smeijsters *et al*., 1994; Graeser *et al*., 1996). The steep increase in *shmt* transcript that we observed parallels this well, but is offset such that it peaks relative to 18S rRNA just as the parasites are starting to replicate their DNA and is maintained throughout this period. It is not yet clear why we see a higher *shmt*/*dhfr-ts* ratio in HB3 compared with Dd2 over this period. One explanation might be that, as Dd2 appears to be more efficient at folate salvage than HB3 (Wang *et al*., 1997a), it is less reliant on serine conversion for its supply of 1-C units. This is because the principal form of folate derived from the host is 5-methyl THF (Baker *et al*., 1994), the methyl group of which can contribute to the 1-C pool (Asawamahasakda and Yuthavong, 1993). The gametocyte mRNA that we analysed was from parasites mainly at the later (IV/V) stages of development. It has been reported that, in *P. falciparum*, mature gametocytes have a full diploid content of DNA (Janse *et al*., 1988), and the high level of *shmt* transcript that we also saw in such parasites may be associated with

the replication that has occurred during differentiation from the haploid asexual stages and/or the further replication that will occur rapidly during meiosis shortly after ingestion by the mosquito.

An increase in *dhfs-fpgs* expression is also seen during the trophozoite stage, which diminishes as the parasites move into schizogony. This may reflect the increased need for folate cofactors during replication being met partly by biosynthesis of THF (as opposed to folate salvage) via DHFS. However, there is very little increase over the same period in the low level of mRNA for *pppk-dhps*, which encodes two additional activities required for the folate biosynthetic route. A more likely explanation for the increase in *dhfs-fpgs* levels might therefore be that the concomitant FPGS activity must be elevated to provide sufficient polyglutamation activity for folate moieties, whether acquired by synthesis or salvage. In other organisms, this modification provides much improved substrates for folate pathway enzymes compared with their monoglutamated counterparts (Krumdieck *et al*., 1992), and folate in *P. falciparum* has also been shown to be polyglutamated (Krungkrai *et al*., 1989).

A key question concerns the relative importance in *P. falciparum* of the biosynthetic pathway leading to the formation of folate (involving GTPCH, DHNA, PPPK, DHPS and DHFS activities), compared with salvage of preformed folate from the host *in vivo* or from tissue culture medium. *In vitro* labelling experiments with folate precursors and folate itself (Krungkrai *et al*., 1989) suggest that both pathways are used, but that salvage may be the predominant route under normal culture conditions, where folate is plentiful. Our data would be broadly consistent with this, in that transcripts for enzymes involved with biosynthesis (particularly *pppk-dhps*) are expressed at a low level, whereas those involved with interconversion of folate cofactors (particularly *dhfr-ts* and *shmt*) are generally at higher levels. However, we find no evidence for any upregulation of biosynthetic activity at the transcriptional level in response to removing the supply of exogenous folate from either Dd2 or HB3 for extended periods. We also observed that the absence of one of the two 256 bp repeat sequences in the 5′ UTR of HB3 *dhfr-ts* mRNA appeared not to alter its expression level relative to that of Dd2 under normal culture conditions.

Another stress situation to which parasite transcription might be expected to respond is challenge by drugs that inhibit the encoded enzyme activities. We monitored whether combined treatment of parasites with the anti-DHFR inhibitor PYR and the anti-DHPS inhibitor SDX would result in upregulation of these or other folate pathway genes to counter the reduction in active enzyme concentration by the drugs. However, again, we found no evidence in either Dd2 or HB3 that the drug-challenged parasites can feed back to the transcription system in this way. This apparent inability to respond to antifolate drug challenge by upregulation of the target enzyme transcripts would be advantageous from the point of view of treatment; however, we note that, when cancer cells are challenged with antifolates, *ts* mRNA levels stay the same, similar to what is observed here, but levels of the protein are increased by up to 40-fold (Chu *et al*., 1993; Keyomarsi *et al*., 1993). This emphasizes the need now to investigate what is occurring at the translational level in the parasite. With respect to novel drug targets, our data suggest that the SHMT enzyme might provide a potent point of inhibition, given its pivotal role in supplying 1-C units to the thymidylate cycle and the evidence presented here that its transcript level is markedly increased across the erythrocytic cell cycle in anticipation of DNA synthesis.

Experimental procedures

Parasite lines

Plasmodium falciparum lines (K1, HB3 and Dd2; Brooks *et al*., 1994; Wang *et al*., 1997a and references therein) were grown in group O+ erythrocytes in standard RPMI-1640 medium and supplements as described (Read and Hyde, 1993), except that 0.5% Albumax-I (Invitrogen) was substituted for human plasma and 5 μg ml^{-1} hypoxanthine was added to cultures. For certain experiments, custom-synthesized RPMI 1640 lacking folate and pABA (Invitrogen) was used (Wang *et al*., 1997b).

Quantitative RT-PCR and diagnostic PCR

In vitro cultures with parasitaemias of ≈5% were exposed to 0.05% saponin in phosphate-buffered saline (PBS) to lyse the erythrocytes, and the liberated parasites were pelleted for RNA extraction using the RNeasy mini kit (Qiagen). An on-column DNase I digestion was carried out using the RNase-free DNase kit (Qiagen). Total RNA extracted from a 10 ml culture (with 1 ml of whole blood) was resuspended in 50 μl of RNase-free water, and 3 μl was used for a 20 μl reverse transcription reaction from an oligo-$(dT)_{15}$ primer using an avian myeloblastosis virus-based first-strand cDNA synthesis kit (Roche) according to the manufacturer's instructions.

Gene-specific PCR used oligonucleotide pairs for the *P. falciparum* folate genes *gtpch*, *pppk-dhps*, *dhfs-fpgs*, *dhfr-ts* and *shmt* (Table 1). For the bifunctional genes *pppk-dhps* and *dhfr-ts*, primer pairs were designed to each of the separate domains, whereas for *dhfs-fpgs*, only a single pair was made, as both activities span the single functional domain of this molecule (Salcedo *et al*., 2001). The primers also encompassed introns where present (*dhps*, *pppk*, *dhfs-fpgs*, *shmt*) to control for genomic DNA contamination. PCR conditions for each gene were initially optimized in a series of experiments using a genomic DNA template. PCRs contained 200 ng each of flanking primer, 200 μM each dNTP, 2.5 mM $MgCl_2$, 2 U of *Taq* polymerase (Promega) per 100 μl of master mix. Template concentrations were based on the relative

amount of 18S rRNA in each preparation, as determined by qRT-PCR (see below) to ensure comparable starting levels across different experiments. Cycling parameters were as follows: a precycle at 95°C for 5 min was followed by 30 cycles of 95°C for 30 s, 30 s at the annealing temperature (see Table 1) and 72°C for 2 min with a final cycle of 72°C for 10 min. Sampling was carried out at two-cycle intervals, at the end of elongation phase, without disrupting the cycle. All samples derived from a given batch of RNA were processed at the same time and analysed on 1% agarose gels. Ethidium bromide concentration, gel volume, loading volume and exposure time were kept constant throughout the experiments to minimize any variability in band intensities.

For analysis of the 5′ UTR of the *dhfr-ts* gene, PCR was carried out as above using primers C599 (5′-GTACAG TTTTTTATTGGTTTCTTAATTG and C597 (5′-GCTATTTGGG AATACAAAAATGGCTAG), with an annealing temperature of 50°C.

Derivation of genomic standard curves

PCR cycling parameters were optimized for each gene studied, and standard curves were constructed using a 10-fold dilution series of a 0.5 mg ml^{-1} stock of K1 genomic DNA as template. A series of identical 50 μl PCR reaction mixes was set up for each gene, and sample tubes were removed at two-cycle intervals as described above. Changes in ethidium fluorescence during the exponential phase of PCR were used to calculate the threshold cycle (Ct) for each gene studied, i.e. the first cycle at which a fluorescent band of the correct size is detectable above the background. Quantification is based on the principle that Ct is inversely proportional to the logarithm of the initial copy number of the gene (Higuchi *et al*., 1993). The derivation of the standard curves for *shmt* and 18S rDNA (*ssu*) is shown in Fig. 2A and B; similar curves were derived for all other genes. Independent probes designed for each of the two domains of the bifunctional genes *pppk-dhps* and *dhfr-ts* acted as internal controls for reproducibility.

Normalization to 18S RNA

Although the genomic standard curves enabled absolute quantification of gene transcripts derived from a given batch of RNA, comparison of data among different samples and experiments was carried out by normalizing to the transcript levels of asexual 18S rRNA (SSU) of *P. falciparum*. This also obviated the need to quantify the scarce RNA samples before RT-PCR. Gene-specific primers *ssuRT* and *ssuFor* plus *ssuRev* were used in the reverse transcription reactions and PCRs respectively (Table 1), and rRNA was quantified by relating threshold cycles to the previously derived 18S rDNA genomic standard curve.

Synchronization assay

Cultures at 5% haematocrit and 5% parasitaemia were synchronized as described (Read *et al*., 1993) with two treatments of 5% sorbitol solution 24 h apart, with a further treatment carried out if necessary, to give parasites with an age range of ± 2 h. Sampling of synchronized cultures began 48 h after the last sorbitol treatment at ring, early trophozoite, late trophozoite and schizont stages.

Folate depletion assay

Red cells from Dd2 and HB3 cultures synchronized as above were centrifuged at 3000 *g* for 5 min, and the pellets were washed twice in sterile PBS to remove residual folate from the medium. Half of each culture was resuspended in complete RPMI 1640, while the other half was resuspended in custom RPMI 1640 supplied free of folic acid and pABA (Invitrogen), to which glucose, hypoxanthine and gentamicin had been added (Wang *et al*., 1997b). After a 48 h incubation period to increase parasitaemia, sampling was carried out at the ring, trophozoite and schizont phases (10 ml cultures of each stage), and folate gene expression was quantified as described above.

Pyrimethamine-sulphadoxine assay

The susceptibilities to SDX-PYR (stock containing 4 mg ml^{-1} SDX, 80 μg ml^{-1} PYR in dimethyl sulphoxide, DMSO) of Dd2 and HB3 parasite lines were characterized using an *in vitro* [^{3}H]-hypoxanthine uptake assay as described previously (Wang *et al*., 1997b). Comparable growth inhibition doses for each strain were calculated based on their corresponding 48 h IC_{50} values. Parasite lines set up in RPMI were synchronized with two or three sorbitol treatments as described above, and SDX-PYR in a 50:1 (w/w) ratio was added 24 h after the last sorbitol treatment (Dd2: 2 μg ml^{-1} SDX, 40 ng ml^{-1} PYR; and HB3: 0.4 μg ml^{-1} SDX, 8 ng ml^{-1} PYR). Control cultures for each strain were identical except that the drug solution was replaced with the same volume of tissue culture-grade DMSO. Sampling commenced after a 24 h period, and corresponded to schizont ≈ 24–28 h, ring ≈ 36–40 h, early trophozoite ≈ 46–50 h and late trophozoite stages ≈ 60–64 h after incubation with the drug. RNA extraction was then carried out as described above.

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