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# Insights into the *P. y. yoelii* hepatic stage transcriptome reveal complex transcriptional patterns

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**Abstract**

During their complex life cycle, malaria parasites adopt morphologically, biochemically and immunologically distinct forms. The intra-hepatic form is the least known, yet of established value in the induction of sterile immunity and as a target for chemoprophylaxis. Using *Plasmodium yoelii* as a model we present here a novel approach to the elucidation of the transcriptome of this poorly studied stage. Sequences from *Plasmodium* were obtained in 388 of the 3533 inserts (11%) isolated from liver stages cDNA obtained from optimized cultures with high yields. These corresponded to a total of 88 putative *P. yoelii* genes. The majority of the transcribed genes identified, code for predicted proteins of as yet unknown function. The RT-PCR analysis carried out for 29 of these genes, confirmed expression at the hepatic stage and provided evidence for complex patterns of genes transcription in the distinct stages found in the mosquito and vertebrate host. The results demonstrate the efficacy of the approach that can now be applied to further detailed analysis of the hepatic stage transcriptome of *Plasmodium*.


*Keywords:* Transcriptome; *Plasmodium yoelii*; Liver stages; Stage-specific transcripts

## 1. Introduction

The unicellular malaria parasites shuttle between the mosquito and the vertebrate hosts, adopting morphologically and biochemically distinct forms, implying complex regulation of the predicted 6000 genes to adapt to very distinct environments. Indeed, from a morphological and biological point of view, there are major differences between the gametes, the oocysts and sporozoites of the sexual cycle in the mosquito, or between these stages and the two distinct asexual rapidly dividing forms occurring first in the hepatocyte and thereafter in the red blood cell of the vertebrate. There are wide differences in parasite numbers depending on the stage, the least numerous being the hepatic forms, which might explain

*Abbreviations:* RTase, reverse transcriptase; MgS, midgut sporozoite; SgS, salivary gland sporozoite; LSc, liver stages parasites from cultures; LSv, liver stages parasites from in vivo; BSt, blood stage parasites; "s", sense transcription; "a", anti-sense transcription

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why they remained little studied for so long. Despite their scarcity, the characteristics of the intra-hepatic transcription are of considerable value from (a) a fundamental biological point of view, for e.g., the intriguing chronobiology of the dormant forms, (b) a malaria control point of view since it is the only stage against which sterile immunity can be induced in humans [1–4], and (c) a therapeutic point of view, as it is biochemically distinct from other stages rich in unique pathways that constitute ideal prophylactic targets as it precedes the pathogenic phase. Therefore, the elucidation of the pattern of gene expression during these hepatic stages is of considerable fundamental and practical value. Molecular studies of liver stages, however, have been difficult to undertake as severe technical and methodological limitations restricted access to the material [3].

In the present work we elaborated a strategy for the analysis of the hepatic stage transcriptome by capitalizing on four recent breakthroughs: the availability of the genome sequence of several *Plasmodium* species, that of the mouse genome, progress in high throughput sequencing, and the development of improved culture conditions of liver stages that lead to markedly higher rates of infection of hepatocytes. We selected *P. yoelii* as a model to examine the feasibility and the output of this approach, because this species is the most widely employed for vaccine and drug development in preclinical models and the life cycle can be easily handled in the laboratory. The first results presented here, demonstrate the validity of the approach.

## 2. Materials and methods

### 2.1. P. y. yoelii hepatic stage culture

Inbred 6–8 weeks female C57BL/6 (H-$2^b$) mice (Charles River, France) were housed and used with approval from the Pasteur Animal Welfare Committee. Hepatocytes were prepared by perfusion of mice liver following a two-step enzymatic protocol [5], with carefully pre-selected Collagenase H batches in a $CaCl_2$ buffer and purified on a 60% Percoll gradient at 2000 rpm for 2 min. Preparations with viability determined by blue trypan exclusion >90%, were suspended in Williams medium E (Gibco, Life Technologies, France) supplemented with 0.2% BSA (Sigma–Aldrich, France), 10% foetal calf serum, 1% penicillin–streptomycin (Gibco, Life Technologies, France), 1% spite (Sigma–Aldrich, France), and 1% L-glutamine. Cells were then seeded at a density of $7 \times 10^4$ per well, in 8-chamber LabTEK permanox slides (Nalge Nunc International, Naperville, Ill.). Sporozoites were obtained by dissection following Ozaki Method [6] from *P. y. yoelii* clone 1.1-infected *Anopheles stephensi* (14 days post-gametocyte infection), and kept in William's medium supplemented with 10% foetal calf serum and 1% penicillin–streptomycin, at 4 °C until use. Only batches with >15,000/20,000 sporozoites per mosquito were employed. Fifty thousand sporozoites in a volume of 200 μl William's medium (Gibco, Life Technologies, France) supplemented with dexamethasone ($10^{-7}$ M) were added per well 24 h after cell plating. Infected hepatocyte cultures were centrifuged as described previously [7] and incubated at 37 °C in 5% $CO_2$ atmosphere for 3 h. The medium was carefully removed and 300 μl of supplemented William's with 3.5 g/l glucose was added to each well. Cultures were incubated at 37 °C in 5% $CO_2$ atmosphere and the medium changed daily. The growth of liver stages was monitored under a phase-contrast inverted microscope. Cells were harvested 48 h after infection only from a selected culture where the prevalence of high-infected hepatocytes was high (≥3%) and the parasitized cells were homogeneously 40–50 μm in size, and kept at −80 °C in RNA Later Buffer (Qiagen, Germany). Two control wells per slide were used to enumerate liver stage parasites using FITC-labelled anti-HSP70 antibody [8], and the residual sporozoites using FITC-labelled anti CS mAb NYS1. All experiments and procedures performed using animals conformed to the French Ministry of Agriculture Regulations for Animal Experimentation (1987).

### 2.2. RNA purification

Messenger RNA was purified from *P. yoelii yoelii* clone 1.1-infected primary mouse (C57Bl/6) hepatocyte cultures seeded in seven 8-well LabTEK culture plates. The cultures were harvested at 48 h post-sporozoites addition, when the parasites were at sub-mature schizont stage (Fig. 1). In the preparation selected, each well contained 70,000 hepatocytes

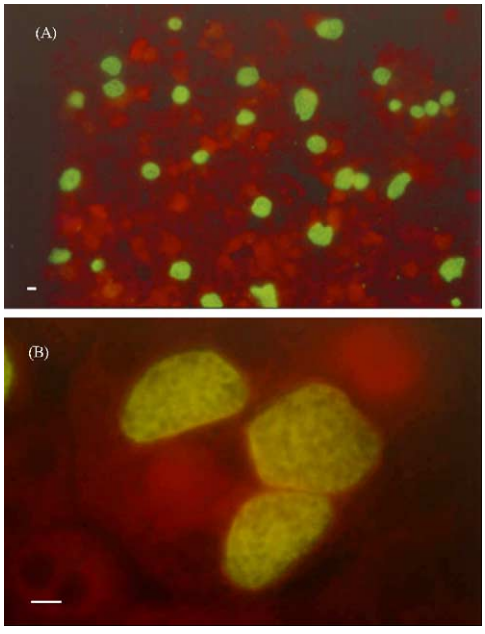


Fig. 1. *Plasmodium yoelii* liver stages employed in this study. IFAT of liver stages in primary hepatocytes culture, performed with antibodies directed against *Plasmodium* HSP70-2, showing the high proportion of parasites that were highly homogeneous in size and close to full maturation though still developing. Panel A: magnification 40×. Panel B: magnification 100×. Scale bar of both panels: 10 μm.