Short communication

A genetic analysis of *Plasmodium falciparum* RNA polymerase II subunits in yeast

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ABSTRACT

RNA polymerase II is an essential nuclear multi subunit enzyme that transcribes nearly the whole genome. Its inhibition by the alpha-amanitin toxin leads to cell death. The enzyme of *Plasmodium falciparum* remains poorly characterized. Using a complementation assay in yeast as a genetic test, we demonstrate that five *Plasmodium* putative RNA polymerase subunits are indeed functional *in vivo*. The active site of this enzyme is built from the two largest subunits. Using site directed mutagenesis we were able to modify the active site of the yeast RNA polymerase II so as to introduce *Plasmodium* or human structural motifs. The resulting strains allow the screening of chemical libraries for potential specific inhibitors.

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Plasmodium falciparum (Pf) is causing malaria [1]. Several drugs exhibit antimalarial activity that target a limited set of enzymatic activities (recently among them the artemisinin derivatives). The parasitic genome was sequenced [2]. The RNA polymerase II (RNAPII) is a complex of 12 subunits and transcribes most of the nuclear genome in eukaryotic cells. The genes encoding its subunits are named RPB1–12 in the yeast *Saccharomyces cerevisiae* (Sc), and will be henceforth referred to as Sc1–Sc12. It is the natural target of alpha-amanitin [3], which blocks RNAPII elongation leading to its degradation *in vivo* [4], ultimately resulting in cell death. Both human (Hs) and Pf RNAPII are sensitive to alpha-amanitin [5]. Therefore, a drug that would selectively inhibit the parasite's enzyme might be of therapeutic value.

In this study, we address the functional relevance of the homology-based candidates for Pf RNAPII subunit coding sequences in Sc. The potential subunits were identified in databases (see Table 1 and Sup Table 1). The coding sequences of the 10 smallest subunits were cloned into a pGEN vector allowing their expression under the control of a PGK promoter (see Sup Table 2A). The ability of putative RNAPII subunits of Pf to replace their Sc counterparts was tested using complementation assays. The corresponding Hs subunits were tested in parallel as controls [6,7]. The complementation assay was performed by transforming each yeast strain, defective for a particular RNAPII subunit gene, with a pGEN

derivative allowing the expression of either the same autologous subunit, or its putative Pf and Hs heterologous homologs (for details see Sup. Data, Materials and Methods). The yeast strains are listed in Sup Table 2B.

Pf4 can replace Sc4 *in vivo*. The DD105 yeast strain lacking Sc4 was viable, although growing very slowly (–), Fig. 1A). When transformed with pGEN Sc4, normal growth was restored (Sc4, Fig. 1A). The expression of both Pf4 and Hs4 efficiently replaced Sc4 as judged from the size of the colonies (Pf4 and Hs4, Fig. 1A) at 30 °C on rich medium. The same efficiencies were observed at 20 °C, while, at 37 °C, neither Pf4 nor Hs4 could replace Sc4 (see Sup. Fig. 2).

Pf5 can replace Sc5 *in vivo*. Sc5 is an essential protein, as confirmed by the inability of the YSF11 strain (see Sup. Table 2B), bearing an interrupted chromosomal RPB5 gene, to grow in its absence (not shown [8]). When transformed with pGEN Sc5, this strain grows (Sc5, Fig. 1B). The expression of Pf5 but not Hs5 in place of Sc5 yielded viable complemented yeast (Sup. Fig. 1: red colonies in Pf5), which showed a reduced growth rate at 30 °C on rich medium (Pf5, Fig. 1B). By contrast, at 20 °C, a nearly WT phenotype was observed, while at 37 °C, Pf5 was essentially inactive (see Sup. Fig. 3).

Pf7 can replace Sc7 *in vivo*. As Sc7 is an essential protein, the D208–4b strain, bearing an interrupted chromosomal RPB7 gene (see Sup. Table 2B), cannot grow in its absence (not shown [9]). D208–4b yeast strain grows when Sc7 expression was restored (Sc7, Fig. 1C). Hs7 replaced Sc7 at 30 °C on rich medium (Hs7, Fig. 1C), Pf7 did not restore a completely wild-type phenotype under the same conditions (Pf7, Fig. 1C). At 20 °C, the difference in colony sizes was

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Table 1
Complementation results for Pf and Hs RNAPII subunits.

Subunit	PlasmoDB: ID N	Identity %	Complementation	Subunit	Identity %	Complementation
Pf3	PF11130c	21	–	Hs3	34	–
Pf4	PFB0245c	16	+	Hs4	19	+
Pf5	PF13.0341	39	+	Hs5	36	–
Pf6	PFC0155c	34	–	Hs6	47	+
Pf7	PF10.0269	33	+	Hs7	43	+
Pf8	PFL0665c	29	–	Hs8	30	+
Pf9	PFA0505c	14	+	Hs9	37	+
Pf10	PF07.0027	51	–	Hs10	71	+
Pf11	PF13.0023	28	–	Hs11a	47	–
Pf12	MAL13P1.213	28	+	Hs12	34	+

considerably reduced (see Sc7, Pf7 and Hs7 [Sup. Fig. 4](#)). By contrast, at 37 °C, Pf7 was essentially inactive, although both Sc7 and Hs7 similarly supported yeast growth (see [Sup. Fig. 4](#)).

Pf9 can replace Sc9 *in vivo*. YVV9 (lacking Sc9 expression) is a viable, slow-growing strain (–), [Fig. 1D](#)). When transformed with pGEN Sc9, the yeast cells grew much faster (Sc9, [Fig. 1D](#)). The expression of Pf9 and Hs9 efficiently replaced their yeast counterpart (Pf9 and Hs9, [Fig. 1D](#)) at 30 °C on rich medium. The same was true when growth took place at 20 or 37 °C (see [Sup. Fig. 5](#)).

Pf12 can replace Sc12 *in vivo*. As Sc12 is also an essential protein, the YGVS020 strain, that bears an interrupted chromosomal RPB12 gene (see [Sup. Table 2B](#)), cannot grow in its absence (not shown [\[10\]](#)). This yeast strain grows when Sc12 expression is restored (Sc12, [Fig. 1E](#)). Both Pf12 and Hs12 replace Sc12 at 30 °C on rich medium (Pf12 and Hs12, [Fig. 1E](#)). At 20 °C, there was no difference in colony size (see Sc12, Pf12 and Hs12 [Sup. Fig. 6](#)). At 37 °C, neither Pf12 nor Hs12 was fully active, as reflected by the smaller colony size (see [Sup. Fig. 6](#)).

Pf3, Pf6, Pf8, Pf10, Pf11 subunits are not able to replace the corresponding Sc subunits. The complementation assays performed with the pGEN vectors expressing Pf3, Pf6, Pf8, Pf10, Pf11, using the D138-

1, JAY444, YGVS043, YGVS072 strains, respectively, did not yield viable colonies (data not shown). The presence of pGEN-encoded mRNAs was verified by RT-PCR, and in all cases the expected transcripts were detected (see [Sup. Fig. 7](#)). The assay in the case of Pf10 was the genetic drift. Loosing the balancer plasmid that carries both the ADE2 and the RPB10 genes, yields viable red colonies, provided the Sc10 protein can be replaced by the pGEN encoded candidate (for example Hs10, see [Sup. Fig. 1](#)). We analyzed any small colony that may have failed to turn red, and had to finally conclude that Pf10 was not functional in yeast.

We summarize our results in [Table 1](#). We have demonstrated that five of these proteins are functional in the yeast cell: Pf4, Pf5, Pf7, Pf9 and Pf12. This is the first formal evidence that these proteins are *bona fide* functional polymerase subunits. Pf9 is the most functional subunit since even at 37 °C no phenotype was observed. Although dispensable for growth under optimal conditions, the Sc9 subunit is very important for elongation in extreme conditions [\[11\]](#). When compared to Sc9, the Pf9 protein exhibits a 120-residue C-terminal extension that does not impair its functions in yeast (see [Sup. Fig. 5](#)). The homology score (14%, [Table 1](#)) is low, demonstrating a poor correlation between this indication and the functionality

Fig. 1. Five *Plasmodium* subunits are functional in yeast. Yeast strains were either transformed with a pGEN vector allowing the expression of a given subunit, as indicated on the corresponding sector of each Petri dish. (–) Refers to the cases where the subunits are not essential. Two independent viable colonies were streaked on YPGlu rich medium and allowed to grow at 30 °C for two to three days. (A) DD105 strain was transformed or not (–) with pGEN Sc4, pGEN Pf4, pGEN Hs4. (B) YSF11 strain was transformed with pGEN Sc5, pGEN Pf5. (C) D208–4b was transformed with pGEN Sc7, pGEN Pf7, pGEN Hs7. (D) YVV9 strain was transformed or not (–) with pGEN Sc9, pGEN Pf9, pGEN Hs9. (E) YGVS020 was transformed with pGEN Sc12, pGEN Pf12, pGEN Hs12.

Fig. 2. RNAPII active center modification. (A) Schematic representation of the Sc1 subunit derivatives that were tested. The line on the top represents the wild-type peptidic sequence (Sc1), where the six residues shown with their coordinates have been mutated as indicated below in colours. In Sc1(Pf1)a five residues were mutated. Similarly, five residues were mutated in Sc1(Hs1)a. Sc1(Pf1)b is a chimeric molecule in which a segment spanning residues 640–815 was replaced by a segment spanning the orthologous residues 796–971 from the Pf1 sequence. In Sc1(Hs1)b the same sequence was replaced by residues 663–838 from Hs1. (B) The JAY47 yeast strain was transformed with a pJAY483 expression vector, either empty ($\emptyset$) or allowing the expression of a Sc1 derivative, as indicated on the sectors of each Petri dish. The untransformed JAY47 was streaked as well (–). A representative colony was streaked on both a supplemented minimal medium as indicated (His, Ade, Leu, Ura, Trp) with either galactose (Gal) or glucose (Glu) as a fermentable carbon source. The yeast cells were allowed to grow at 30 °C for four days.

of a protein *in vivo*. Pf4 and Pf7 are fully functional RNAPII subunits in yeast at 20 °C. Sc4 and Sc7 form a dimer that is bound to the minimal RNAPII complex, mainly via Sc4, and are involved in mRNA trafficking [12]. Pf4 is poorly conserved (see Table 1 or Sup. Fig. 2), though efficient *in vivo*. Hs4 complemented strain exhibits a strong phenotype at 37 °C (see Sup. Fig. 2), which is in contrast with a previously published result [6]. This discrepancy is most probably related to the fact that the incubation temperature was more carefully controlled in the present work, arousing more confidence in the present result, in agreement with others [13]. Pf5 and Pf12 can replace their Sc homologs, and are present within the three types of nuclear RNA polymerase: RNAPI, RNAPII and RNAPIII. The three polymerase activities are simultaneously assayed. The small Pf12 subunit appears to be functionally well conserved, since only at 37 °C is a phenotype observed (see Sup. Fig. 6). It can be noticed that Hs12 exhibits a moderate phenotype at 37 °C. Strikingly, Pf5 is fully functional at 20 °C, but very sensitive to enhanced temperatures (see Sup. Fig. 3). This seems to be a common property, since Pf4, Pf5, Pf7, Pf9 and Pf12 all exhibit nearly wild-type growth at 20 °C, in sharp contrast with their respective behaviour at higher temperatures.

Though Pf4 and Pf9 exhibit homology scores as low as 15%, they are functional in distantly related species. By contrast, Pf10 and Pf6, respectively, 51% and 36% homologous to their yeast counterparts are not functional. We investigated the reason behind this observation (data not shown). There are two domains that differ between Sc10 and Pf10 (residues 17–42 and 52–70) (see Sup. Fig. 9). We created chimeric Sc10 proteins in which we exchanged independently both domains. When assayed in complementation, none of these recombinant was viable. At least two major changes are responsible for the lack of function of the Pf10 protein in yeast.

In the case of Pf6, we noticed a 15-residue peptidic insertion within a very conserved domain (Pf6 114–128, Sup. Fig. 10). We

inserted it within the Hs6 protein at the same place. When assayed, the resulting recombinant turned out to be viable, although with a thermosensitive phenotype (data not shown). The presence of this insert within the Pf6 subunit does not explain why it does not work in yeast. It may be worth stressing that Sc5, 6, 8, 10 and 12 are shared by RNAPI, RNAPII and RNAPIII, so that the lack of viability of complemented strains may be due to only one polymerase activity defect.

We were able to amplify both Pf1 and Pf2 coding sequences, but not to clone them in any vector, preventing any complementation test. We decided to focus on the alpha-amanitin binding domain within the RNAPII active center that is mainly located on the largest subunit. We thus constructed the recombinant Sc1 derivatives described in Fig. 2A. The Sc1 residues that are in close contact with the toxin [14] were mutated when different to those present at the same position in either the *Plasmodium* or the human sequence, in order to locally mimic either the *Plasmodium* parasite or its human host RNAPII, generating Sc1(Pf1)a and Sc1(Hs1)a, respectively. Two chimeras were built using unique restriction sites flanking this region (see Fig. 2A and Sup. Fig. 11). A 176-residue sequence of Sc1 was replaced by either the homologous plasmodium or the human sequence, generating Sc1(Pf1)b and Sc1(Hs1)b, respectively.

The ability of these constructs to replace the wild-type Sc1 was tested in the JAY47 strain, under glucose mediated repression of the chromosomal GAL10-RPB1 chimeric gene as shown in Fig. 2B (see Sup. Data, Materials and Methods). Representative colonies of each transformation were streaked on supplemented galactose medium and all are viable (YNB Gal HALUW, Fig. 2B). On glucose medium, very different growths were observed (YNB Glu HALUW, Fig. 2B). The JAY47 strain, whether transformed or not with a pJAY483Δ empty vector (– and $\emptyset$, Fig. 2B), could not grow. The expression of Sc1 restores growth (Sc1, Fig. 2B). The mutation of five residues

within the RNAPII active center [Sc1(Pf1)a and Sc1(Hs1)a, Fig. 2B] did not prevent growth on glucose medium. In contrast, the swapping of the 176-residue peptide yielded non functional molecules [Sc1(Pf1)b and Sc1(Hs1)b, Fig. 2B] (see also Sup. Fig. 8). This does not formally rule out that the full-length Pf1 or Hs1 proteins may be functional, but strongly argues against this possibility. The edges of the swapped peptide are located in closely homologous regions and only substitutions can be responsible for the phenotype (see Sup. Fig. 11). When assaying the full length Pf1 and Hs1 proteins, a large number of additional changes are likely to add to the phenotype.

The alpha-amanitin binding domain of the active center of RNAPII is shared by both largest subunits, Sc1 and Sc2. The 3D structure of the yeast RNAPII complexed with amanitin has been resolved [14]. Most of the residues involved in the binding of the toxin are located within the largest subunit, in a domain previously genetically identified [15]. Both Sc1(Pf1)a and Sc1(Hs1)a constructs are viable, although Sc1(Pf1)a exhibited a slightly decreased growth rate (data not shown). The side chains of the six residues pointing to the lumen of the active site, this is not unexpected and confirms the validity of the established 3D model and genetic data. The availability of three yeast strains differing only by a few residues (Sc1(Pf1)a and Sc1(Hs1)a and the Sc1 control) within the RNAPII active site will potentially be useful for screening for inhibitors.

This work represents to our knowledge the first attempt to genetically characterize the RNAPII of *Pf*. In addition, we constructed viable mutated enzymes that exhibit modified active sites within the alpha-amanitin binding region. The resulting yeast strains may prove useful to design convenient tests for the identification of new potential antimalarial drugs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2010.12.013.

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