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An unusual recent expansion of the C-terminal domain of RNA polymerase II in primate malaria parasites features a motif otherwise only found in mammalian polymerases

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Abstract

The tail of the enzyme RNA polymerase II is responsible for integrating the diverse events of gene expression in eukaryotes and is indispensable for life in yeast, fruit flies and mice. The tail features a C-terminal domain (CTD), which is comprised of tandemly repeated Y_1-S_2-P_3-T_4-S_5-P_6-S_7 amino acid heptads that are highly conserved across evolutionary lineages, with all mammalian polymerases featuring 52 identical heptad repeats. However, the composition and function of protozoan CTDs remain less well understood. We find that malaria parasites (genus *Plasmodium*) display an unprecedented plasticity within the length and composition of their CTDs. The CTD in malaria parasites which infect human and non-human primates has expanded compared to closely related species that infect rodents or birds. In addition, this variability extends to different isolates within a single species, such as isolates of the human malaria parasite, *Plasmodium falciparum*. Our results indicate that expanded CTD heptads in malaria parasites correlates with parasitism of primates and provide the first demonstration of polymorphism of the RNA polymerase II CTD within a single species. The expanded set of CTD heptads feature lysine in the seventh position (Y_1-S_2-P_3-T_4-S_5-P_6-K_7), a sequence only otherwise seen in the distal portion of mammalian polymerases. These observations raise new questions for the radiation of malaria parasites into diverse hosts and for the molecular evolution of the RNA polymerase II.

Keywords

protozoa; Plasmodium; Apicomplexa; transcription

Introduction

Malaria is responsible for a significant burden on human health and economic productivity in the developing world. The malaria parasite *Plasmodium falciparum* has co-evolved with humans for millennia and displays sophisticated patterns of gene expression that enable the parasite to multiply in several distinct morphological stages in remarkably different environments, including clonal replication in the liver and circulating red blood cells of its mammalian host as well as both sexual and asexual replication within its mosquito vector. Despite these major changes in gene expression, analyses of the completed sequence of the *P. falciparum* genome have resulted in the report of a surprising “paucity” of identifiable transcription factors (Aravind, Iyer et al. 2003) or well-characterized domains that are found in transcription factors shared from budding yeast to humans (Iyer, Anantharaman et al. 2008). Nonetheless, the basic aspects of transcription in *Plasmodium*, mediated by RNA

polymerase II, appear to be similar to those described for metazoans, including 5′ capping, polyadenylation, splicing and chromatin modifications (Aravind, Iyer, Wellems, and Miller

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Data deposition footnote:
Sequences generated by this study: RNA Polymerase II CTD (regions R2 and R3)

	Accession Code:	Source
P. gallinaceum RNA polymerase II (whole)	EU840284	Sanger
P. berghei isolate ANKA	EU827171	Edinburgh
P. berghei isolate NK65	EU827172	Edinburgh
P. yoelii yoelii 17X	EU827173	Edinburgh
P. yoelii yoelii 33X	EU827174	Edinburgh
P. yoelii killicki	EU827175	Edinburgh
P. yoelii nigeriensis	EU827176	Edinburgh
*P. yoelii (*new subspecies)	EU827177	Edinburgh
P. vinckei VIBA CyO	EU827178	Edinburgh
P. vinckei VIBA Cy P1	EU827179	Edinburgh
P. vinckei lentum 194ZZ	EU827180	Edinburgh
P. vinckei (new subspecies)	EU827181	Edinburgh
P. reichinowi	EU850397	Sanger
P. vivax isolate ONG	EU840276	MRA-341G
P. vivax isolate Pakchong	EU840277	MRA-342G
P. vivax isolate Nicaragua	EU840278	MRA-343G
P. vivax isolate Panama	EU840283	MRA-340G
P. cynomolgi isolate Smithsonian	EU840279	MRA-351G
P. cynomolgi isolate Cambodian	EU840280	MRA-579G
P. cynomolgi isolate Bastianelli	EU840281	MRA-350G
P. fragile	EU840282	MRA-352G
P. falciparum isolate FCR3	EU827182	MRA-731
P. falciparum isolate D6	EU827183	MRA-285
P. falciparum isolate RO33	EU827184	MRA-200
P. falciparum isolate 7G8	EU827185	NIH
P. falciparum isolate HB3	EU827186	MRA-155
P. falciparum isolate Santa (St.) Lucia	EU827187	MRA-331
P. falciparum isolate FCC-2	EU827188	MRA-733
P. falciparum isolate D10	EU827189	NIH
P. falciparum siolate K1	EU827190	MRA-159
P. falciparum isolate W2	EU827191	MRA-157
P. falciparum isolate DD2	EU827192	MRA-156
P. ovale	EU887536	NYU

Edinburgh = R. Carter, University of Edinburgh

MRA = Malaria Research and Reference Reagent Resource Center (www.mr4.org)

NIH = National Institutes of Health

NYU = J. Carlton, New York University

2003). Hence, attention is rapidly turning to the study of the role of RNA polymerase II in transcriptional regulation to more fully understand the basic biology of malaria parasites.

RNA polymerase II is a central component of a complex apparatus responsible for controlling the basic biochemical steps required for mRNA production, including transcription initiation, capping, elongation, splicing, and polyadenylation (Howe, 2002;Zorio and Bentley 2004;Proudfoot, Furger et al. 2002). These diverse processes are integrated by the tail of the molecule, referred to as the C-terminal domain (CTD) of the protein (Carty and Greenleaf 2002). The CTD, essential for gene expression in animals and fungi, is comprised of a tandem array of heptapeptide repeats, featuring a signature amino acid sequence: Y_1-S_2-P_3-T_4-S_5-P_6-S_7 (Corden, 1990;Allison, Moyle et al. 1985). In yeast, rodents and humans, these repeats coordinate transcriptional events by undergoing reversible phosphorylation at the 2nd and 5th serines of the heptad (Corden, 2007;Schroeder, Schwer et al. 2000;Licatalosi, Geiger et al. 2002), with more recent data implicating the 7th serine as critical for transcriptional elongation (Chapman, Heidemann et al. 2007;Egloff, O'Reilly et al. 2007). The combinatorial phosphorylation patterns create novel docking platforms for the sequential recruitment of specific transcription factors involved in initiation, capping, splicing and termination.

The length of the CTD varies in different organisms and roughly tracks with genome size. For example, the CTD of the microsporidian *Encephalitozoon cuniculi* contains 15 repeats while a CTD containing 52 repeats is present in all mammalian species thus far described (Chapman, Heidemann, Albert, Mailhammer, Flatley, Meisterernst, Kremmer, and Eick 2007;Egloff, O'Reilly, Chapman, Taylor, Tanzhaus, Pitts, Eick, and Murphy 2007). The perfect conservation of the mammalian CTD demonstrates the strict conservation of this portion of RNA pol II within broad evolutionary lineages. The length of the CTD is essential for RNA polymerase II stability, transcriptional efficiency and ultimately cell viability. For example, the CTD of the budding yeast *Sacharomyces cerevisiae* consists of 26 heptad repeats and truncation to 17, 14, 13 or 11 repeats leads to respective reductions in specific transcript levels to 58%, 8%, 5% and 2% of that found in wild type cells (Liao, Taylor et al. 1991). Further reduction of the repeat length to 8–10 repeats yields cells that are temperature-sensitive, and truncation to less than eight repeats is lethal. Similarly, 30 of the 52 hepatads are necessary for viability in mice (Meininghaus, Chapman et al. 2000) and mutants with 31–39 heptads exhibit growth defects (Bartolomei, Halden et al. 1988;Litingtung, Lawler et al. 1999). The importance of CTD length is reflected in the fact that this region of the protein is strictly conserved in closely related organisms, and no variability within an individual species in the number of heptad repeats has been observed.

Throughout evolution, the 7th position of the conserved heptad repeat displays the most heterogeneity in higher eukaryotes (Guo and Stiller 2005;Liu, Greenleaf et al. 2008). In yeast the serine in this position is non-essential, with substitution of alanine tolerated (Stiller, McConaughy et al. 2000). Intriguingly, mammals, including rodents and primates, feature seven to nine non-consensus Y_1-S_2-P_3-T_4-S_5-P_6-$\mathbf{K}_7$ heptads near the distal end of the CTD (Barron-Casella and Corden 1992;Guo and Stiller 2005). In mammals, while 20 of the first 25 heptads contain a serine in the 7th position, only 6 of the last 27 heptads do, and 8 of the last 17 heptads all contain a lysine in this position. In human cell lines, however, these lysine-containing repeats are dispensable, with polymerases consisting of only consensus repeats supporting normal growth and viability of cells (Chapman, Conrad et al. 2005). A further study by Chapman et al. indicated that polymerases featuring an excess of lysine heptads fail to support cell viability (Chapman, Heidemann, Albert, Mailhammer, Flatley, Meisterernst, Kremmer, and Eick 2007). Thus the role of the lysine heptads in mammalian polymerases remains an outstanding question in RNA polymerase II biology and awaits functional, biochemical and regulatory characterization.

The completion and release of the genome sequences from several *Plasmodium* species has revealed that this genus – alone outside of mammalian genera-- extensively utilize lysine-containing repeats within their RNA pol II CTDs. This observation raised the question of what role these repeats might play CTD function, and led us to study the composition of the CTD across the *Plasmodium* genus. We find that the number of heptad repeats in *Plasmodium* is remarkably small, with the rodent and bird malaria parasites possessing only eight heptad repeats within their CTDs. Surprisingly, unlike other evolutionary lineages in which heptad numbers are tightly conserved, closely related *Plasmodium* species display very different numbers of heptad repeats. In particular, primate parasites posses an expanded CTD of up to nine additional lysine containing repeats, a motif otherwise only found in the distal half of mammalian polymerases. The expansion appears to have occurred at least twice in evolution, once in a lineage giving rise to *P. falciparum* and independently in the lineage giving rise to *P. vivax* and other non-human primate parasites. Strikingly, the plasticity appears to extend to individual species of *Plasmodium*. Different geographical isolates of the human parasites *P. falciparum* and *P. vivax* and the primate parasite *P. cynomolgi* also exhibit significant flexibility in repeat numbers. These findings provide the first examples of polymorphism of the RNA polymerase II CTD within a single species. Taken together, our observations raise new questions for both the evolution of malaria parasites and the RNA polymerase II molecule as well as malaria parasitism of diverse hosts. In addition, as *P. falciparum* and *P. berghei* are genetically tractable systems, this work opens the possibility to elucidating the hitherto cryptic function of lysine heptads in eukaryotes.

Materials and Methods

Amplification of Plasmodium CTDs

Genomic DNA for *P. falciparum, P. vivax and P. simium* strains were obtained from the Malaria Research and Reference Reagent Resource Center (MR4; www.mr4.org) and Dr. X. Su of the National Institute of Allergy and Infectious Disease. Genomic DNA from *P. ovale* was a gift by J. Carlton (New York University, New York City) and genomic DNA for isolates and sub-species of rodent malarias (*P. berghei, P. yoelii* and *P. vinckei*) were from stocks held at the University of Edinburgh by R. Carter (Perkins, Sarkar et al. 2007). Oligonucleotide primers complementary to sequences flanking the first repeat in the CTD and the carboxy-terminus of the largest subunit of RNA polymerase II (*Rbp1*) gene were used to PCR-amplify the CTD. All PCR reactions were carried out on a PTC-2000® Peltier thermal cycler using *Taq* polymerase® (Invitrogen) under the following conditions: 95 °C for 5 min followed by 35–38 cycles of 94 °C for 30 s, 56 °C for 60 s, 60 °C for 1 min, and a final extension step of 65 °C for 5 min. Reaction products were purified and directly sent for automated sequencing by the university core facility. The forward primer that was used to amplify the RNA polymerase II CTD from *P. falciparum* isolates was 5′-CCTAAACCTCAAATTAATCATAATATTTATTCA-3′ and the reverse primer was 5′-CATATTTTCCTTCATTTCGTCCTCGTATAT-3′. The forward primer for *P. vivax* and *P. fragile* RNA polymerase II CTD was 5′ – TCCCC(A/C)TT(C/T)TCTCC(A/T/C)TTTGAT-3′ and the reverse primer was: 5′-CATTTCGTCCTC(C/G)TC(C/T)ATGTTGTA-3′ The forward primer for *P. berghei* RNA polymerase II CTD was 5′-CCAAAACCTCAGATGCAAAATAATATATATTCT-3′ and the reverse primer was: 5′-TTATTCTTCCTGCATCTCCTCTTCATCCAT-3′; forward primer for *P. yoelii yoelii* RNA polymerase II CTD was 5′-CCAAAACCTCAGATGCAAAATAATATATATTCT-3′ and the reverse primer was as above for *P. berghei*.

Tabulation of Repeat Numbers

Repeats were counted manually and defined conventionally, as heptads featuring a serine in the second and fifth position (S_2 and S_5) (Liu, Greenleaf, and Stiller 2008). The lone exception to this rule was made for the repeat YAIASPK in the non-human primate parasite *Plasmodium fragile* as it aligned with the repeat YSITSPK in rodent and human parasites. We note that this definition differs from recent work of Chapman et al. (Liu, Greenleaf, and Stiller 2008) who include phasing in their definition. This group identifies five repeats in *P. yoelii*; whereas we identify eight. Diheptads or heptad pairs refers to two heptad repeats found in tandem with no intervening amino acid residues. A single heptad can be counted as part of two diheptads. For example, three consecutive heptad repeats would constitute two diheptads.

Results

Plasticity of the CTD within the Genus *Plasmodium*

Previously, two laboratories described a greater number of CTD heptad repeats in *P. falciparum* when compared to the rodent malarias *P. berghei* and *P. yoelii* (Chapman, Heidemann et al. 2008;Egloff and Murphy 2008;Giesecke, Barale et al. 1991), suggesting an unusual variability in CTD structure between these closely related organisms. Since the number of CTD heptad repeats is generally stringently conserved between even distantly related genera, we wondered if the differences between the human and rodent parasites reflected a general plasticity of CTD heptads in the genus *Plasmodium*. To obtain a broad perspective of CTD sequences from throughout the *Plasmodium* genus, we collected sequences from the largest subunit of RNA polymerase II (Rbp1) from parasites infecting humans (*P. falciparum*, *P. vivax* and *P. ovale*), non-human primates (*P. reichenowi*, *P. knowlesi* and *P. fragile*), rodents (*P. berghei* and *P. yoellii*) and birds (*P. gallinaceum*). The sequences were either obtained from genome sequence databases at the NCBI or Sanger Institute, or directly amplified from parasitized blood obtained from field samples. This collection of sequences was then aligned using CLUSTALW, adjusted through visual inspection and repeats tabulated as described in the Materials and Methods.

Analysis of the amino acid sequences from the various species showed that they all maintained the typical, conserved CTD structure consisting of a linker region (R1), the heptad repeat-containing region (R2), and a tail region following the last repeat (R3) (Chapman, Heidemann, Hintermair, and Eick 2008). However, while all of the species possessed the heptad repeat structure typical of CTDs from higher eukaryotes, there was a remarkable degree of variability in the number of repeats in region R2 as well as in the amino acid found in the 7^{th} position of each heptad (Figure 1). This is in stark contrast to the extreme conservation of heptad sequence observed in many other evolutionary lineages. For example, mammals from different orders exhibit no variability in either heptad structure or number of repeats, with all species thus far examined displaying nearly indistinguishable (one amino acid difference between mice and humans) CTDs consisting of 52 identical repeats (Barron-Casella and Corden 1992). Second, the *Plasmodium* CTDs are remarkably short, consisting of only 8 repeats in the rodent and bird parasites, *P. berghei, P. yoellii*, and *P. gallinaceum,* and reaching a maximum of 13–15 repeats in the primate parasites *P. knowlesi*, *P. vivax*, *P. reichenowi* and *P. falciparum*. The exception to this trend is *P. ovale* which possesses only 8 repeats within the CTD. In addition, the different *Plasmodium* species all displayed a propensity toward a non-serine amino acid in the 7^{th} position, and in particular the primate parasites exhibited an expanded set of tandemly arrayed repeats containing lysine in this position. All of the species that display an expanded CTD infect monkeys, apes or humans, thus correlating increased heptad number with parasitism of primates.

When phylogenetic analyses between the various species of *Plasmodium* are overlaid on the CTD data, there is support for two separate expansions of the lysine heptads (denoted by asterisk in Figure 1) in primate parasites. One expansion occurred after *P. fragile*, *P. knowlesi* and *P. vivax* diverged from *P. ovale* and the other expansion occurred in the lineage giving rise to *P. falciparum* and *P. reichinowi*. Codon usage data supports this argument; *P. falciparum* and *P. reichinowi* share single expansion of a codon for the lysine repeats (AAA), whereas the other primate parasites that have undergone expansion (*P. vivax*, *P. knowlesi* and *P. fragile*) feature lysine codon AAG as the common or predominant codon in the repeats. Moreover, the rest of the RNA polymerase II molecule of these parasites is more closely related to the polymerase of the rodent parasite *P. berghei*, which lacks the expanded motifs, than to the primate parasite *P. falciparum*, which features them (data not shown). The separate expansions imply that the increased CTD length provides a selective advantage for parasites infecting primate hosts.

Plasticity of the CTD within a single species of *Plasmodium*

Such extensive plasticity in CTD heptad repeat numbers and composition between closely related species of a single genus has not been described before. We therefore questioned whether the CTD structure might be rapidly diverging within the *Plasmodium* genus, and consequently whether such plasticity might extend to an individual species. For this analysis we took advantage of recent efforts to sequence the genomes of several independent isolates of *P. falciparum* obtained from infected individuals from different malarious regions around the world. The sequence of RNA polymerase II from an isolate from Honduras ((Li, Bzik et al. 1989); NCBI database) and one from Papua New Guinea (D10; Broad Institute database) were analyzed for CTD sequence and structure. Notably, both of these isolates exhibited fewer heptad repeats (Honduras: 14 repeats; D10: 13 repeats) than 3D7 (15 repeats), the reference strain used for the initial genome sequencing project. To determine if the change in repeat number was an artifact of genome sequence assembly, we obtained genomic DNA from both 3D7 and D10 (gift from Dr. X. Su, NIAID), and directly amplified the CTD encoding region of the gene using species-specific PCR primers and sequenced the purified amplicons. We found that both sequences precisely matched the sequences reported in the initial genome nucleotide sequence databases, thus verifying the observed polymorphisms.

To determine the degree of repeat number polymorphism within *P. falciparum*, we extended the analysis to additional isolates from other geographically diverse locations, including 4 African, 5 Asian and 3 American isolates of *P. falciparum* from the Malaria Research and Reference Reagent Resource Center (MR4) of the American Type Culture Collection (ATCC). We amplified the CTD from genomic DNA and directly sequenced the purified amplicons. The results displayed an even greater degree of polymorphism than initially observed, with isolates exhibiting CTD lengths ranging from as few as 12 to as many as 17 repeats (Figure 2A). Both heptad repeats and diheptad repeats (repeats in tandem) were tabulated. The variations appear to weakly cluster geographically. Asian isolates (e.g. DD2, K1 and D10) tend to be shorter (12–13 repeats) while American isolates (St. Lucia, HB3 and 7G8) all have 14 repeats. African isolates tend to be the longest with 15 in 3D7, 17 in FCR3 and 14 in RO33; with the exception of the African isolate D6, which has only 13 repeats. Figure 2B shows a phylogenetic tree of the various *P. falciparum* isolates based on 137 SNPs (Volkman, Sabeti et al. 2007), which indicates the CTD polymorphism tracks with continental Asian and American clades. The one exception is FCR3, which was reportedly isolated from the Gambia, but genetic analysis has suggested might actually be of Asian origin (Volkman, Sabeti, DeCaprio, Neafsey, Schaffner, Milner, Jr., Daily, Sarr, Ndiaye, Ndir, Mboup, Duraisingh, Lukens, Derr, Stange-Thomann, Waggoner, Onofrio, Ziaugra, Mauceli, Gnerre, Jaffe, Zainoun, Wiegand, Birren, Hartl, Galagan, Lander, and Wirth 2007).

We next asked whether two other primate parasites, *P. vivax* and *P. cynomolgi,* exhibit plasticity in CTD repeat numbers similar to *P. falciparum*. As done for the *P. falciparum* samples, using known database sequence and genomic DNA from MR4, we amplified the CTD from several DNA samples and directly sequenced purified amplicons. Similar to *P. falciparum*, we found plasticity of the CTD specific to the lysine-containing, tandemly arrayed heptad repeats (Figure 2A). There is also a geographic correlation to the repeat number with Asian isolates of *P. vivax* (ONG and Pakchong) exhibiting more repeats (15 and 14), respectively than the American (Nicaragua, Panama and Salvador-1) isolates (13 repeats). The Smithsonian isolate of *P. cynomolgi* features one more repeat than the Bastianelli and Cambodian isolates.

To determine if the intra-species polymorphisms in CTD length extend to other *Plasmodium* species, we took advantage of 11 rare samples of genomic DNA obtained from various isolates and sub-species of the rodent malaria parasites *P. berghei*, *P. yoelii* and *P. vinckei* as described in Perkins et al., 2007 (Perkins, Sarkar, and Carter 2007). These rodent parasite species are restricted to West and Central Africa. We observed no differences in repeat numbers either across or within the *P. berghei*, *P. yoelii* or *P. vinckei* species or subspecies (Figure 2A). The number of repeats was also identical to the number reported for the bird parasite, *P. gallinaceum*. These data suggest that the diversity of heptad repeat numbers may be specific to the primate malaria parasites *P. falciparum, P. vivax* and *P. cynomolgi*. In addition, the polymorphisms do not appear to be an artifact of laboratory culture, as seen in budding yeast (Nonet, Sweetser et al. 1987), because fresh uncultured clinical isolates also display plasticity of repeats (unpublished data).

Evolution of the CTD in protozoa

The heptad repeat structure of the CTD is a ubiquitous feature of RNA pol II of metazoa, and it is indispensable for transcriptional activity in these organisms. Among the unicellular eukaryotes, however, some organisms possess RNA pol II enzymes that contain CTDs with recognizable heptads, while others do not, suggesting that the CTD evolved within an ancient organism near this point in the eukaryotic lineage. In light of the remarkable diversity of CTD heptads within the *Plasmodium* genus and within different isolates of *P. falciparum* and *P. vivax*, we investigated whether closely related protozoa possess conserved CTD heptads. In particular we were interested in other apicomplexans, as well as other alveolates such as dinoflagellates and ciliates. Our examination of CTDs from protozoa using conventional genome sequence databases indicated that CTD heptads are also present in the apicomplexans *Babesia bovis; Theileria spp; Toxoplasma gondii* and *Cryptosporidium spp* as well as in the early branching dinoflagellate *Perkinsus marinus*. The apicomplexans and dinoflagellates examined possessed heptad repeats that varied in size from nine in *Toxoplasma* to as many as 24 in *Cryptosporidium*. The 7th position of the heptads varies across organisms with an alanine in *Babesia* and *Perkinsus* and a histidine in *Cryptosporidium*. The two closely related parasites, *Cryptosporidium parvum* and *Cryptosporidium hominis*, possess the same heptad repeat composition and number throughout their CTDs; whereas the murine parasite *Cryptosporidium muris* displays the same number of heptads but utilizes a different heptad composition that relies on histidine and arginine in the 7th position. Thus the remarkable variability observed in repeat numbers in *Plasmodium* may be unique to this genus.

DISCUSSION

The extreme plasticity that we observed for the CTD heptad repeats in *Plasmodium* has not been described for any other eukaryotic species. Moreover, the intra-species plasticity is not observed across any of the rodent parasites we studied. It is possible that the repetitive nature of the DNA sequence encoding the CTD simply leads to frequent duplications and

deletions, and in this regard *Plasmodium* might have a propensity toward replication errors in the absence of purifying selection regulating CTD length. However, the lack of tandem repeats in the ancestral Plasmodium CTD suggest that the amplification observed in primate parasites was not simply the result of random replication errors (i.e. unequal crossover) and instead likely arose from positive selection. Rather than displaying random variation in length and composition, the primate malarias appear to have a specific expansion of a particular heptad, leading to a longer CTD. Further, both phylogenetic analyses and codon-usage bias support the hypothesis that the expansion of the CTD occurred twice in primate parasites – once in the line giving rise to *P. falciparum* and once in a line giving rise to *P. vivax*, and not any non-primate parasites. The fact that these expansion events appear to have occurred independently and in parallel suggests a strong functional basis for the longer CTD. The nature of this selective force in primates remains cryptic and no available data correlates CTD length in human parasites with virulence, drug resistance or any other obvious phenotype.

We did not observe variation in the CTDs of any of the rodent parasites, and their length was identical to that of *P. gallinaceum*, a parasite of birds, suggesting that the non-primate parasites might be fixed in their repeat numbers at eight heptads. In addition, the eight specific heptads found in bird and rodent parasites are also conserved in the CTDs of primate parasites (Figure 1), indicating that this represents the ancestral structure of the CTD prior to the expansion observed in primate parasites. Interestingly, eight repeats is consistent with the minimal number of heptads required for viability in yeast and the minimal number in the mouse RNA pol II to form a secondary structure resembling a full length CTD (Bienkiewicz, Moon et al. 2000;Liao, Taylor, Kingston, and Young 1991). We therefore surmise that the 8 repeats might be the minimal number required for a functional RNA polymerase II, and thus this represents the shortest CTD possible within this genus. However, in yeast the functional unit of the CTD was shown to be pairs of tandemly arranged heptad repeats, and that at least 7 such “diheptads” were required for viability (Stiller and Cook 2004). Thus, it appears that both the number and tandem arrangement of repeats is important for function. It is not yet clear which specific RNA polymerase functions require the tandem arrangement of the heptads, though the tandem arrangement is present in all eukaryotes from yeast to mammals. Notably, the rodent and bird parasites do not possess the minimal number of diheptad repeats required for viability in yeast, while the heptad expansion found in the CTD of primate parasites is specific for tandemly arranged repeats. This expansion greatly increases the number of diheptads (Figure 2A) resulting in CTDs that meet or exceed what is required in higher eukaryotes. In contrast to metazoan CTDs, in which the tandemly repeated structure of the CTD is strongly conserved, heptads are often found in isolation in protists. Hence, the expanded set of perfect tandemly arranged repeats in several Plasmodium species suggests that the CTD-related proteins of these parasites might share functional requirements with those from higher eukaryotes.

Interestingly, the expanded repeats all feature lysine in the seventh position, a motif only otherwise found in mammalian polymerases. The expansion of lysine containing heptads in primate parasites highlights the potential significance of repeats that deviate from the consensus sequence YSPTSPS, particularly in the 7^{th} position. The 7^{th} position of the conserved heptad repeat displays the most heterogeneity in higher eukaryotes (Guo and Stiller 2005;Liu, Greenleaf, and Stiller 2008) and in yeast, the serine in this position is non-essential (Stiller, McConaughy, and Hall 2000). Thus the role of the lysine-enrichment in the distal end of the RNA polymerase II remains an outstanding question in CTD biology (Egloff and Murphy 2008;Phatnani and Greenleaf 2006). It has been hypothesized that lysine containing heptads might confer additional specificity in recruitment of specific proteins to the transcription complex (Phatnani and Greenleaf 2006). Naturally, this raises the question of why plasmodia species utilize lysine containing repeats within their CTDs,

particularly as our analysis indicates that the extensive reliance on lysine containing heptads is unique to *Plasmodium* and not present in other organisms, including other apicomplexan parasites. In the case of *P. falciparum* the lysine (codon: AAA or AAG) enrichment could simply be a natural consequence of selective pressures of its A/T-rich (80%) genome. However, *P. vivax* also displays a similar number of lysine containing repeats but is nearly balanced in its A/T content (57%), implying functional significance for these heptads. In light of recent data concerning lysine modifications on non-histone targets involved in gene regulation, it is possible that the lysines in the CTD might be differentially acetylated, particularly by adjoining transcription factors (e.g. TAF1 subunit of TFIID) (Mizzen, Yang et al. 1996).

Why Plasmodium should evolve a longer CTD in just a few and not all of its mammalian host species is intriguing. It is likely that the expanded lysine heptads reflect individual or small sets of interactions that have been incrementally added to the transcriptional repertoires of primate parasites. One could imagine that the primate-infecting parasites have co-opted functional requirements for transcription shared with higher eukaryotes during co-evolution with host transcription systems. It is less likely, however, that the expanded lysine heptads are themselves interacting with mammalian CTD-related proteins, at least during the erythrocytic stages, as this portion of the parasite life cycle transpires in the aenucleated red blood cell environment.

This work also sheds light on the evolution of the CTD. We find that all Apicomplexa studied possess heptad repeats. By contrast, ciliates lack CTD heptads altogether despite their grouping with apicomplexans within Alveolata. Interestingly, the RNA polymerases of ciliates possess serine-proline (SP) motifs that are enriched within the C-terminus but not elsewhere in RNA polymerase II (Figure 3). A similar pattern of “SP” richness is also seen in the eukaryotic human pathogen *Trichomonas* (Dacks, Marinets et al. 2002), but is not seen in *Giardia* or the kinetoplastids (*Trypanosoma cruzi*, *Trypansoma brucei* and *Leishmania major*). The “SP” motifs are reminiscent of Y_1-S_2-P_3-T_4-S_5-P_6-X_7 heptad motifs found in metazoan and apicomplexan CTDs, suggesting the possibility that they are either a direct result of degeneracy or serve as early, primitive repeats. The ciliate *Stylonchia mytilus*, for instance, features regular, repeating SP motifs that are often preceded by tyrosine residues (e.g. YSP motifs). These observations suggest that that the SP motifs have evolved as specific sites for phosphorylation of RNA pol II. However, the absence of SP motifs or heptads does not necessarily imply the absence of phosphorylation of the RNA pol II; for example, the CTD of *T. brucei* is phosphorylated (Chapman and Agabian 1994).

The study of RNA polymerase II CTDs in protozoa, particularly with the ever expanding number of genome sequences that are becoming available, is providing valuable insights into the evolution of this important protein domain. The fact that different species of *Plasmodium* naturally display variable CTDs, and that both *P. falciparum* and *P. berghei*, haploid single-celled protists, are genetically tractable systems, means that it might be possible to use these organisms to test many of the current hypotheses regarding the roles of heptad repeats in CTD function. Thus *Plasmodium*, an organism of great importance to human health and economic development, could also serve as a model system for understanding the evolution of transcription in eukaryotic biology.

Acknowledgments

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Abbreviations

CTD carboxy-terminal domain or C-terminal domain

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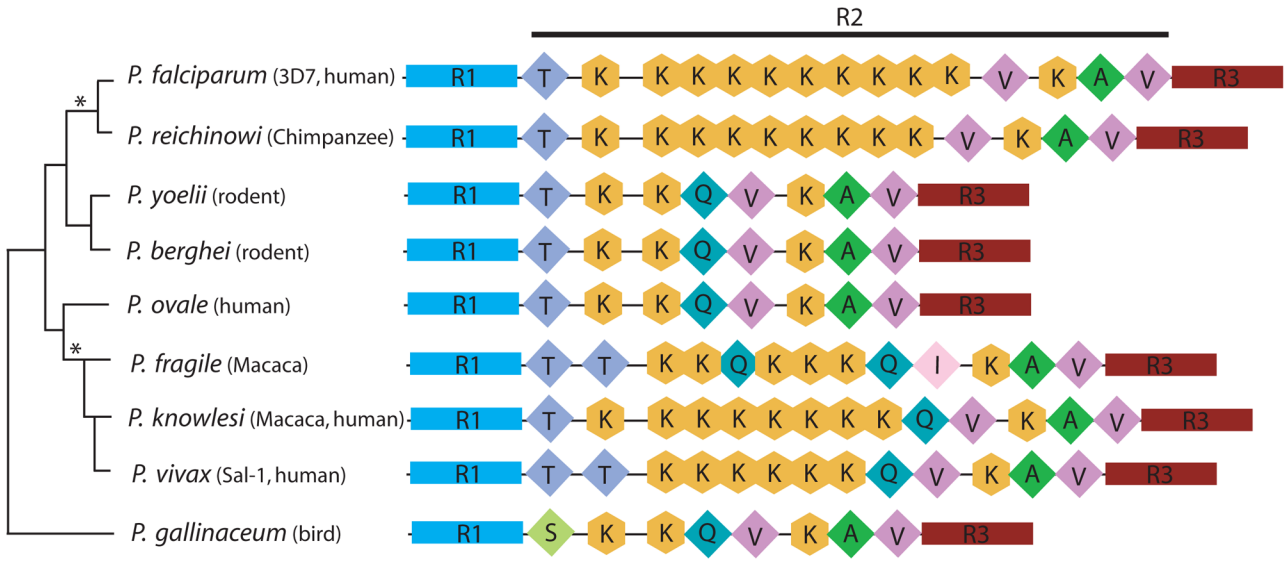
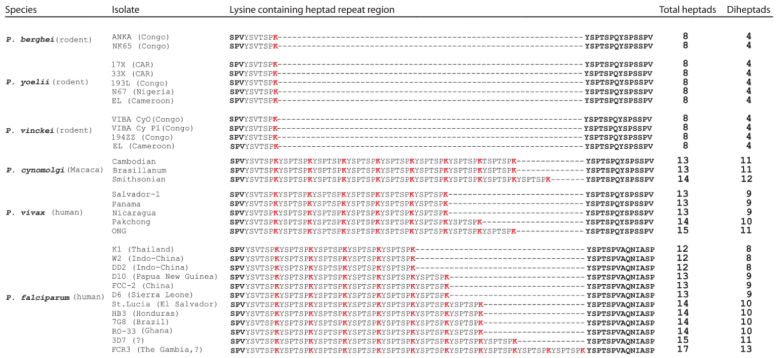


Figure 1. Plasticity of the heptads in the RNA polymerase II CTD across the *Plasmodium* genus

Schematic depiction of the CTD heptads from eight species of *Plasmodium*. The species names are listed at the left of each line along with a tree indicating their evolutionary relationships, constructed using sequences from the three mitochondrial protein-coding genes of the parasites as described in Perkins (2008). R1 is the linker domain, R2 is the heptad-bearing region and R3 is the acidic tail region following the last repeat of the CTD. Diamonds and hexagons symbolize individual heptads, with the amino acid in the 7th position indicated. The plasticity of the CTD is restricted to heptad-containing region, R2. Rodent malaria parasites (*P. yoelii* and *P. berghei*) and the bird parasite (*P. gallinaceum*) exhibit eight repeats with a common stalk (Q,V,K,A,V). Primate malaria parasites feature an expanded set of tandemly arrayed heptads all containing lysine in position 7 of heptads immediately preceding this stalk. Two separate, independent expansions are proposed to have occurred (indicated by asterisks). Sequences of the CTDs drawn from PlasmoDB (www.plasmodb.org) and contigs at the Sanger Institute: *P. berghei*: ANK,PB000038.00.0; *P. yoelii*17xNL: PY03187; *P. vivax* Sal-1: PV095320; *P. knowlesi* Strain H: PKH_082310; *P. falciparum* 3D7: Pfc0805w. *P. gallinaceum:* EU840284 and *P. reichinowi*: EU850397, *P. fragile:* EU840282 and *P. ovale:* EU887536.

Figure 2A



Species	Isolate	Lysine containing heptad repeat region	Total heptads	Diheptads
P. berghei (rodent)	ANKA (Congo)	SPVYSVTSPK----------YSPTSPQYSPSSPV	8	4
	NK65 (Congo)	SPVYSVTSPK----------YSPTSPQYSPSSPV	8	4
P. yoelii (rodent)	17X (CAR)	SPVYSVTSPK----------YSPTSPQYSPSSPV	8	4
	33X (CAR)	SPVYSVTSPK----------YSPTSPQYSPSSPV	8	4
	193L (Congo)	SPVYSVTSPK----------YSPTSPQYSPSSPV	8	4
	N67 (Nigeria)	SPVYSVTSPK----------YSPTSPQYSPSSPV	8	4
	EL (Cameroon)	SPVYSVTSPK----------YSPTSPQYSPSSPV	8	4
P. vinckei (rodent)	VIBA CyO(Congo)	SPVYSVTSPK----------YSPTSPQYSPSSPV	8	4
	VIBA Cy P1(Congo)	SPVYSVTSPK----------YSPTSPQYSPSSPV	8	4
	194ZZ (Congo)	SPVYSVTSPK----------YSPTSPQYSPSSPV	8	4
	EL (Cameroon)	SPVYSVTSPK----------YSPTSPQYSPSSPV	8	4
P. cynomolgi (Macaca)	Cambodian	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKTSPTSPK----------YSPTSPQYSPSSPV	13	11
	Brasillanum	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK----------YSPTSPQYSPSSPV	13	11
	Smithsonian	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK----------YSPTSPQYSPSSPV	14	12
P. vivax (human)	Salvador-1	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK----------YSPTSPQYSPSSPV	13	9
	Panama	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK----------YSPTSPQYSPSSPV	13	9
	Nicaragua	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK----------YSPTSPQYSPSSPV	13	9
	Pakchong	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK----------YSPTSPQYSPSSPV	14	10
	ONG	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK----------YSPTSPQYSPSSPV	15	11
P. falciparum (human)	K1 (Thailand)	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK----------YSPTSPVAQNIASP	12	8
	W2 (Indo-China)	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK----------YSPTSPVAQNIASP	12	8
	DD2 (Indo-China)	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK----------YSPTSPVAQNIASP	12	8
	D10 (Papua New Guinea)	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK----------YSPTSPVAQNIASP	13	9
	FCC-2 (China)	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK----------YSPTSPVAQNIASP	13	9
	D6 (Sierra Leone)	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK----------YSPTSPVAQNIASP	13	9
	St.Lucia (El Salvador)	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK----------YSPTSPVAQNIASP	14	10
	HB3 (Honduras)	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK----------YSPTSPVAQNIASP	14	10
	7G8 (Brazil)	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK----------YSPTSPVAQNIASP	14	10
	RO-33 (Ghana)	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK----------YSPTSPVAQNIASP	14	10
	3D7 (?)	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKTSPTSPK----------YSPTSPVAQNIASP	15	11
	FCR3 (The Gambia,?)	SPVYSVTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPKYSPTSPK YSPTSPVAQNIASP	17	13

Figure 2B

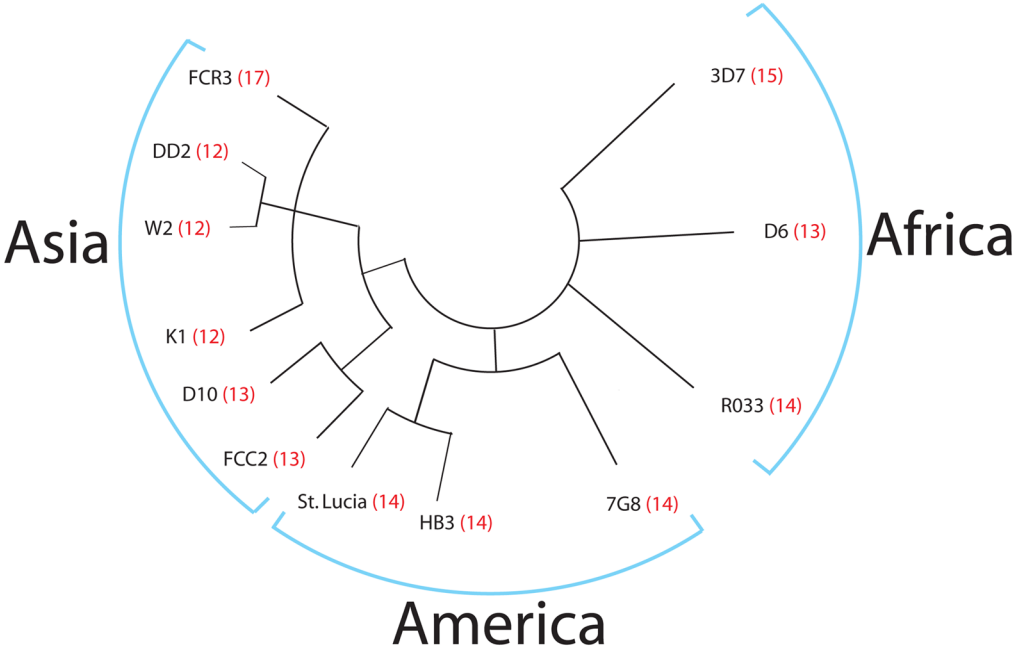


Figure 2. Plasticity of the heptads in the RNA polymerase II CTD across *Plasmodium* species

A. Amino acid sequences of the R2 domain of 2 isolates of *P. berghei*, 5 isolates of *P. yoelii* and 4 isolates of *P. vinckei* (all rodent parasites), 3 isolates of *P. cynomolgi*, 5 isolates of *P. vivax* and 12 geographical isolates of *P. falciparum*. The lysine in the 7th position of each heptad repeat is shown in red. Numbers of total heptads and diheptads are tabulated at the right. B. Phylogenetic tree of various *P. falciparum* isolates based on 137 SNPs and arranged according to geographical origin. The number of heptad repeats found in the CTD of each isolate is shown in red. Adapted from (Volkman, Sabeti, DeCaprio, Neafsey, Schaffner, Milner, Jr., Daily, Sarr, Ndiaye, Ndir, Mboup, Duraisingh, Lukens, Derr, Stange-Thomann, Waggoner, Onofrio, Ziaugra, Mauceli, Gnerre, Jaffe, Zainoun, Wiegand, Birren, Hartl, Galagan, Lander, and Wirth 2007).

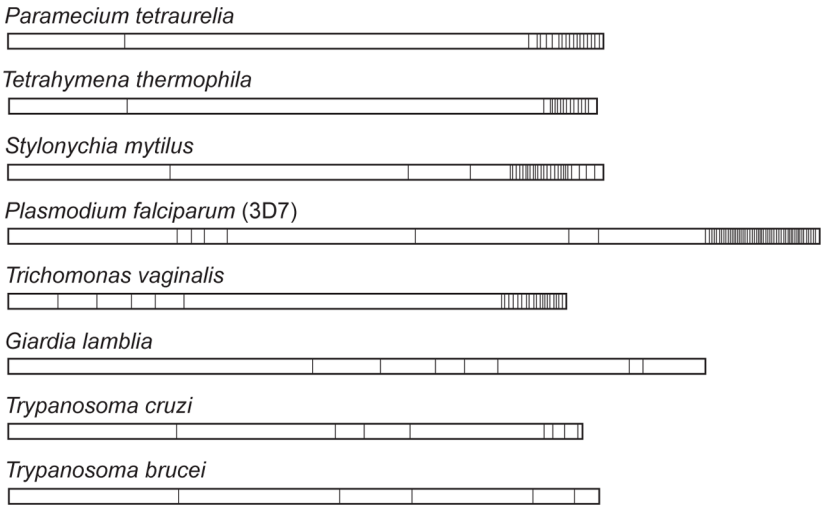


Figure 3. Enrichment of "SP" motifs in the tails of RNA Pol II from eight different protozoan species

Serine-proline (SP) motifs that represent potential phosphorylation sites are marked with vertical lines **(|).** Sequence accession numbers are: *Stylonchia mytilus*: AAK00313.1; *Paramecium tetraurelia*: CAI39063.1; *Tetrahymena thermophila*: GI accession: 118348890; *Trypanasoma brucei*: P17545; *Trypanasoma cruzi*: XP_812569; *Trichomonas vaginalis*: TVU20501; *Giardia lamblia*: XP_001704218