

Short technical report

A densely overlapping gene fragmentation approach improves yeast two-hybrid screens for *Plasmodium falciparum* proteins

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ABSTRACT

Use of the yeast two-hybrid assay to study *Plasmodium falciparum* protein–protein interactions is limited by poor expression of *P. falciparum* genes in yeast and lack of easily implemented assays to confirm the results. We report here two methods to create gene fragments – random fragmentation by partial DNase I digestion and generation of densely overlapping fragments by PCR – that enable most portions of *P. falciparum* genes to be expressed and screened in the yeast two-hybrid assay. The PCR-based method is less technically challenging and facilitates fine-scale mapping of protein interaction domains. Both approaches revealed a putative interaction between PfMyb2 (PF10_0327) and PFC0365w. We developed new plasmids to express the proteins in wheat germ extracts and confirmed the interaction in both the split-luciferase assay and in co-purification experiments with glutathione-S-transferase and HA-tagged proteins. The combination of improved yeast two-hybrid screening approaches and convenient systems to validate interactions enhances the utility of yeast two-hybrid assays for *P. falciparum*.

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The yeast two-hybrid assay is a powerful tool for the discovery and characterization of protein–protein interactions. Since it was first reported more than 20 years ago [1], the assay and its many variations have been integral to the analysis of thousands of proteins. However, the yeast two-hybrid assay has not been widely used to study *Plasmodium* protein–protein interactions, due in large part to the poor expression of *P. falciparum* genes in *Saccharomyces cerevisiae* [2]. AT-rich *P. falciparum* sequences resemble *S. cerevisiae* motifs that specify cleavage and polyadenylation of the nascent RNA [2]. These sequences cause *P. falciparum* mRNAs expressed in yeast to be truncated prematurely and result in degradation by the mRNA surveillance pathway [2,3]. To improve expression of *P. falciparum* genes in yeast, we previously reported the identification of yeast strains with mutations in the mRNA processing pathway [3]. Although these strains are useful for expressing *P. falciparum* proteins for functional studies and pair-wise yeast-two-hybrid assays, they grow more slowly and mate less efficiently than parental strains, and are not optimal for library-based yeast two-hybrid screens. Thus, alternative methods are needed to improve the yeast two-hybrid assay for *P. falciparum*. Here we report the development of two approaches to generate fragments of *P. falciparum* genes for use in the yeast two-hybrid assay. These fragmentation approaches

enabled identification of interactions that could not be detected with full-length genes and are generally applicable to other systems as well.

Large-scale sequencing of clones from *P. falciparum* yeast two-hybrid libraries revealed gene fragments from a wide range of genes and detected no biases [4]. Since these libraries included only fragments that were expressed in yeast, most *P. falciparum* genes appear to contain sequences that can be expressed even if the full-length gene cannot. To more fully investigate this possibility, we tested two methods to fragment *P. falciparum* genes using *PfMYB2* (PF10_0327, 2745 nucleotides, 74% AT) as a test case. *PfMYB2* encodes a 915-amino acid protein with two MYB DNA-binding domains at the N-terminus. Based on the presence of the MYB domains, PfMyb2 has been proposed to function as a helix–turn–helix transcription factor [5].

Random fragments of *PfMYB2* were created using partial DNase I digestion in the presence of manganese, which promotes the formation of blunt-ended fragments and fragments with one base 5' or 3' overhangs. After polishing the ends with T4 DNA polymerase, we ligated double-stranded DNA oligos to the fragments; the oligos were homologous to the sequences flanking the multiple cloning site in the yeast two-hybrid DNA binding domain plasmid pOBD.111 to enable cloning by recombination in yeast. The DNA was then size-fractionated on a Sephacryl S400 column to remove small fragments and unligated oligos. Fragments larger than ~300 base pairs (bp) were PCR-amplified and cloned into pOBD.111 by gap repair in the yeast strain R2HMet [4,6]. Yeast expressing *PfMYB2* fragments were selected on medium lacking tryptophan

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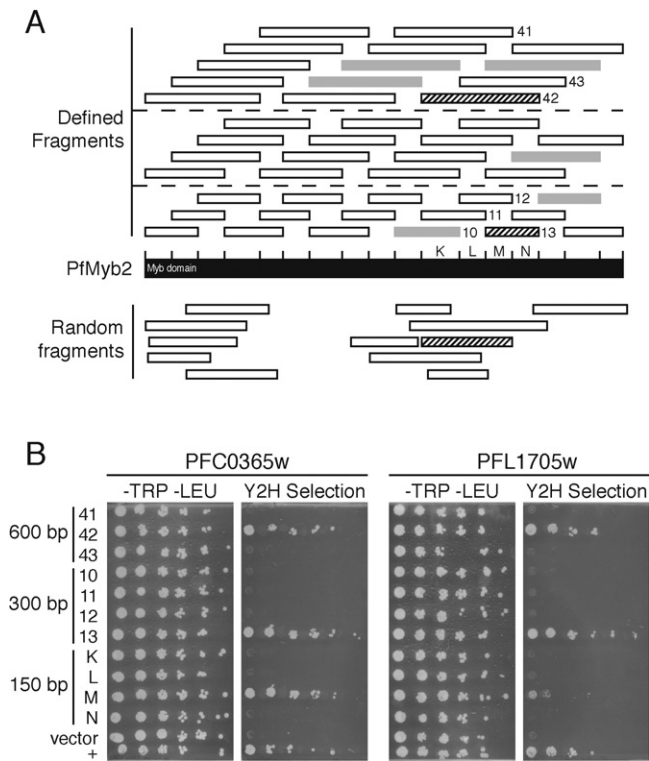


Fig. 1. Gene fragmentation approaches to improve *P. falciparum* yeast two-hybrid screens. (A) Gene fragments generated by partial DNase I digestion (bottom) and PCR (top). Black bar represents *PfMyb2* (PF10_0327). Bars below *PfMYB2* represent fragments generated by partial DNase I digestion of *PfMYB2* that were expressed in yeast. Tick marks above the bar indicate the positions of PCR primers, which were spaced at ~150-bp intervals. Short bars above *PfMyb2* represent 300-, 450-, and 600-bp fragments; dashed lines separate fragments by size. All fragments were cloned into pOBD.111 by homologous recombination in the yeast strain R2HMet and tested for expression of the fragment by growth on synthetic dropout (SD) medium lacking methionine; the *MET2* gene is fused to the 3' end of the *PfMYB2* fragment and is expressed only if the *PfMYB2* fragment is also expressed. White bars represent gene fragments that were expressed, whereas gray bars represent those that were not. Numbers and letters indicate fragments that were retested for interaction with *PFC0365w* and *PFL1705w* in part B; letters designate 150-bp fragments. The twelve DNase I-generated fragments and all 600-bp defined fragments were screened against a *P. falciparum* yeast two-hybrid library. Striped bars indicate fragments that interacted with both *PFC0365w* and *PFL1705w* in the yeast two-hybrid assay. (B) Confirmation of yeast two-hybrid interactions with *PfMYB2* and mapping of the *PfMYB2* protein interaction domain. *PfMYB2* 150-bp fragments K–N, 300-bp fragments #10–13, and 600-bp fragments #41–43 were cloned into pOBD.111 in the yeast strain R2HMet. Similarly, the *PFC0365w* and *PFL1705w* fragments that interacted with *PfMYB2* fragment #42 were PCR-amplified and inserted into pOAD.102 by homologous recombination in the yeast strain BK100. Yeast harboring DNA-binding domain and activation domain plasmids were allowed to mate and then grown on SD medium lacking tryptophan and leucine to select diploid cells containing both plasmids. Yeast were grown to mid log phase, diluted to an OD_{600} of 1.0, serially diluted 5-fold in dH_2O , and plated on SD medium lacking tryptophan and leucine or yeast two-hybrid selection medium (SD medium lacking tryptophan, leucine, methionine, uracil, and histidine and containing 1 mM 3-amino-4,5-triazole (3-AT); 3-AT is a competitive inhibitor of the yeast two-hybrid reporter enzyme His3 that is added to growth medium to inhibit background yeast growth).

and methionine. Because the *MET2* gene is fused to the 3' end of the *PfMYB2* fragment, growth of yeast in the absence of methionine indicates that the *PfMYB2* fragment is expressed. Twelve fragments with diverse start and end points were identified (Fig. 1A, below bar). These fragments included most of the *PfMYB2* gene except for an ~400 bp region near the center. Based on these data, it was not possible to determine if this region could not be expressed in yeast or if too few clones were evaluated.

Because of the incomplete coverage of the random fragments and the technical challenges of the partial DNase I digestion, we

investigated an alternative approach to fragment *PfMYB2*. DNA oligos were designed to hybridize at ~150 bp intervals on *PfMYB2* and used to create a mini-library of densely overlapping gene fragments (Fig. 1A, top, Supplementary Table 1). Every possible 300-, 450-, and 600-bp fragment was PCR amplified and cloned into pOBD.111 by *in vivo* homologous recombination. Fragments expressed in yeast were identified by growth on medium lacking methionine. Of the 45 fragments tested, 39 fragments were expressed at sufficient levels to enable yeast growth. These fragments covered the entire *PfMYB2* gene, indicating that the region for which no random fragments were obtained can be expressed and that the lack of random fragments in this region was due to sampling error.

To identify *P. falciparum* proteins that interacted with *PfMYB2*, all 600-bp defined fragments and the 12 random fragments were screened against a *P. falciparum* activation domain library [4]. Screens were performed manually as described in supplemental methods. Both defined fragment #42 and a random fragment with nearly the same start point interacted with fragments of *PFC0365w* and *PFL1705w*; these genes were the only ones found by both a defined and a random fragment. To confirm these interactions and to further delineate the domains of *PfMyb2* implicated in the interactions, we recloned the *PFC0365w* (nucleotides 133–615, amino acids 45–205) and *PFL1705w* (nucleotides 286–639, amino acids 95–213) fragments into the activation domain vector in fresh yeast and tested their ability to interact with a series of *PfMYB2* fragments that overlapped with fragment #42. Both *PFC0365w* and *PFL1705w* interacted with *PfMYB2* fragment 600-bp #42 and the 300-bp fragment #13 (nucleotides 1936–2235, amino acids 645–745). The 150-bp fragment M (nucleotides 1936–2085, amino acids 646–695) interacted with *PFC0365w* to the same extent as larger fragments #42 and #13, suggesting that the entire *PFC0365w* binding region was encoded by this fragment. In contrast, *PFL1705w* interacted poorly with fragment M and not at all with adjacent fragment N, which was encompassed by fragment #13, suggesting that the binding domain spans the junction of fragments M and N. This experiment delineated a 50 amino acid region of *PfMyb2* as the binding domain for *PFC0365w* and a 100 amino acid region as the binding domain for *PFL1705w*. Surprisingly, fragment #12, which contained fragment M and fragments #41 and #43, which included the sequences in fragments M and #13, did not interact with either *PFC0365w* and *PFL1705w*.

A second problem with yeast two-hybrid screens of *P. falciparum* genes is the lack of convenient systems to validate interactions. *P. falciparum* proteins express poorly in most heterologous systems, antibodies are not available for the vast majority of *P. falciparum* proteins, and generating transgenic parasites expressing epitope-tagged proteins for colocalization and co-purification experiments is still time-consuming and technically challenging. To facilitate confirmation of yeast two-hybrid interactions, we utilized wheat germ extracts, which have been reported to improve the expression of a wide range of *P. falciparum* proteins [7–9]. Four new plasmids were developed to express *PfMyb2*, *PFC0365w*, and *PFL1705w* in the wheat germ *in vitro* translation system for use in the split-luciferase assay and co-purifications with glutathione S transferase (GST)- and HA-epitope tagged proteins (Fig. 2A).

In the split-luciferase assay, proteins of interest are fused to N- and C-terminal fragments of luciferase. If the proteins interact, the N- and C-terminal luciferase fragments associate to create a functional luciferase enzyme [10]. Though a number of variants of this system have been described, for these experiments we used fragments of firefly luciferase that were optimized to yield a high signal to background ratio and sufficient enzymatic activity to be easily measured [11]. We first tested the system using the *P. falciparum* interacting proteins PFE1350c (nucleotides 25–405, amino acids 9–135) and PFC0255c (nucleotides 10–411, amino acids 4–137) [4,12], which were fused to the N- and C-FLUC

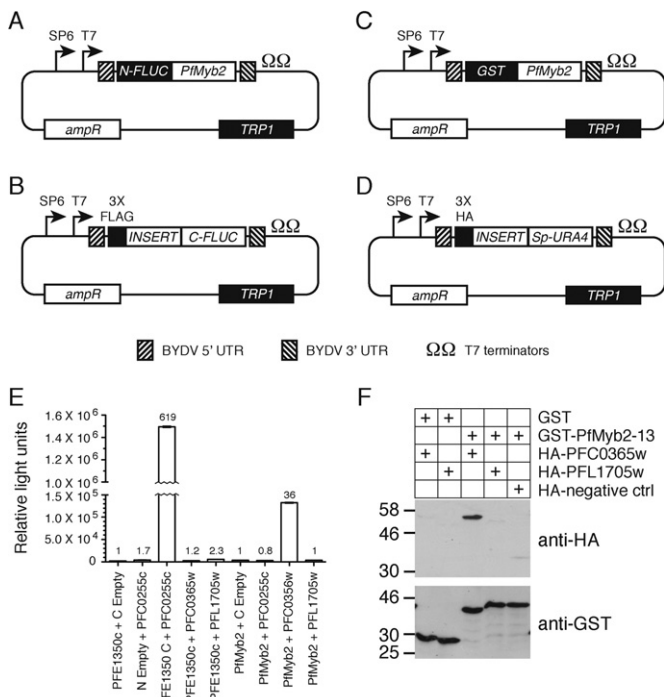


Fig. 2. Confirmation of PfMyb2 interactions. (A–D) New vectors for expressing fusion proteins in wheat germ extracts. The SP6 and T7 promoters, 5' and 3' UTRs from barley yellow dwarf virus (BYDV), and T7 transcription terminators from plasmid pF3A WG (BYDV) (Promega) were cloned into plasmid p424-GPD [20] in place of the *GPD1* promoter and *CYC1* terminator sequences to generate plasmid p424-BYDV-UTR. DNA sequences encoding the N-terminal firefly luciferase fragment (*N-FLUC*, amino acids 1–398) (A), C-terminal firefly luciferase fragment (*C-FLUC*, amino acids 394–550) (B), glutathione S transferase (*GST*) (C), and the *URA4* gene from pOAD.102 (*Sp-URA4*) (D) were inserted into p424-BYDV-UTR by homologous recombination. The *C-FLUC* and *Sp-URA4* plasmids contain the multiple cloning region and flanking sequences from pOAD.102 to facilitate cloning of fragments identified in yeast two-hybrid screens, plus sequences encoding three copies of the FLAG or HA epitope tags, respectively. Similarly, the *N-FLUC* and *GST* plasmids contain the multiple cloning region and flanking sequences from pOBD2 to facilitate transfer of genes from yeast two-hybrid DNA binding domain plasmids. All plasmids confer resistance to ampicillin in *E. coli* and have unique restriction sites for traditional cloning approaches. (E) Confirmation of the PfMyb2–PFC0365w interaction in the split-luciferase assay. *PfMYB2* 300-bp fragment #13 and *PFE1350c* were cloned into the *N-FLUC* plasmid, whereas the *PFC0365w* and *PFL1705w* fragments from the yeast two-hybrid screen and *PFC0255c* were cloned into the *C-FLUC* plasmid. All proteins were expressed using the TNT® SP6 High-Yield Wheat Germ Protein Expression System according to the manufacturer's protocol (Promega). Expression was verified by western blotting with anti-FLAG (Sigma) and anti-N-terminal firefly luciferase (Santa Cruz Biotechnology) antibodies (Supplementary Fig. 1). Equal volumes of *N-FLUC* and *C-FLUC* in vitro translation reactions were added to PBS supplemented with 1% BSA and protease inhibitors (Roche), incubated at 18 h 4 °C with occasional mixing, and assayed for luciferase activity in triplicate. Graph shows the average relative light units (RLU) and standard deviation from each sample after subtracting background luminescence from the buffer only sample. The ratio of the RLU from each combination of *N-FLUC* and *C-FLUC* fusions relative to the *N-FLUC* fusion plus *C-FLUC* negative control is shown above the bar. (F) Confirmation of the PfMyb2–PFC0365w interaction by co-purification. *PfMYB2* 300-bp fragment #13 and the *PFC0365w* and *PFL1705w* fragments from (E) were cloned into the expression vectors shown in (C) and (D), respectively. *GST*- and *HA*-tagged proteins were expressed in the TNT® SP6 High-Yield Wheat Germ Protein Expression System (Promega). A 3-fold excess volume of the in vitro translation reactions of the *HA*-tagged proteins was added to *GST* or *GST*-*PfMYB2* in PBS supplemented with 1% BSA, 0.1% Tween 20, and protease inhibitors (Roche). After overnight incubation at 4 °C with rotation, *GST* tagged proteins were purified using glutathione beads and eluted by boiling in SDS sample buffer. Samples were subjected to SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with antibodies against *HA* (top) and *GST* (bottom). Proteins were imaged by chemiluminescence (GE Health Sciences). Molecular weight markers (sizes in kDa) are shown to the right of each blot.

domains, respectively. The fusion proteins and the negative controls were expressed in wheat germ extracts, mixed, incubated overnight, and assayed for luciferase activity. The combination of *N-FLUC*-PFE1350c and PFC0255c-*C-FLUC* produced a strong signal that was more than 600-fold greater than the background signal from *N-FLUC*-PFE1350c and the *C-FLUC* negative control. Neither PFC0255c-*C-FLUC* plus the *N-FLUC* negative control, nor the combination of *N-FLUC*-PFE1350c with either PFC0365w-*C-FLUC* or PFL1705w-*C-FLUC* produced luciferase activity more than 2.3-fold above the signal from *N-FLUC*-PFE1350c plus the *C-FLUC* negative control. *N-FLUC*-PfMyb2 fragment #13 yielded a strong signal when mixed with PFC0365w-*C-FLUC* (36-fold greater than *N-FLUC*-PfMyb2 plus the *C-FLUC* negative control), thus confirming a physical interaction between PfMyb2 and PFC0365w. However, the combination of *N-FLUC*-PfMyb2 and PFL1705w-*C-FLUC* resulted in only background levels of luciferase activity.

We obtained similar results in co-purification experiments using *GST*-tagged PfMyb2 fragment #13 and *HA*-epitope tagged PFC0365w and PFL1705w. *HA*-PFC0365w copurified with *GST*-PfMyb2 but not *GST* alone, whereas *HA*-PFL1705w did not co-purify with either protein. Thus, we were able to validate the interaction between PfMyb2 and PFC0365w in two independent assays. In addition, the *S. cerevisiae* proteins with greatest homology to PfMyb2 and PFC0365w (Cef1 and Prp19) also interact with each other [13,14]. Thus, the interaction between PfMyb2 and PFC0365w is of high confidence. In contrast, the interaction between PfMyb2 and PFL1705w was not confirmed in either secondary assay and there is no evidence for an interaction between Cef1 and the *S. cerevisiae* ortholog of PFL1705w, Mot2. The *Saccharomyces* Genome Database (www.yeastgenome.org) currently lists 16 studies that used a variety of techniques to identify 62 proteins that physically interacted with Cef1 or Mot2. None of these studies found an interaction between Cef1 and Mot2, nor is there any data to suggest a potential genetic interaction between these genes in yeast. While we cannot dismiss the possibility that the split-luciferase and the *GST* pulldown results are false-negatives, the interaction is not supported by other evidence and is of lower confidence.

Surprisingly, there was no detectable homology between PfMyb2 and Cef1 in the PFC0365w/PRP19 binding domains, even though the yeast orthologs of PfMyb2 and PFC0365w also interact. In fact, the only significant homology between Cef1 and PfMyb2 was in the first 233 amino acids that encode the Myb DNA-binding domain motifs (49% identical, 70% similar). This result was unexpected because protein interaction interfaces are generally thought to evolve more slowly, since changing residues in one protein would require compensating changes in the binding partner. The interaction between PfMyb2 and PFC0365w demonstrates that this is not always the case. A similar observation has been made for three pairs of interacting proteins from *Caenorhabditis elegans* [15]. Despite the lack of homology between PfMyb2 and Cef1 C-terminal to the MYB DNA-binding domain, the fact that PfMyb2 binds to the *P. falciparum* PRP19 homolog suggests that PfMyb2 is the functional homolog of Cef1. Since Cef1 is primarily involved in RNA splicing, we propose that PfMyb2 functions in a similar manner and is unlikely to act as a transcription factor.

In summary, we developed improved methods to identify and confirm *P. falciparum* protein–protein interactions in the yeast two-hybrid assay. Though these approaches were designed to address problems specific to *P. falciparum*, densely overlapping fragmentation and the split-luciferase assay are likely to be useful for studying protein–protein interactions in other species as well. Of the two methods investigated, the defined fragmentation approach was less technically challenging to implement, ensured that all regions of the target gene were tested for expression, and facilitated mapping of the interaction domain. The use of fragments not only enabled all regions of *PfMYB2* to be expressed in yeast, but also

was critical for identifying protein–protein interactions. The interaction between PflMyb2 and PFC0365w was extremely sensitive to both the start and end points of the fragments expressed in the yeast two-hybrid vectors. Furthermore, some fragments that completely overlapped with the protein interaction domain failed to yield positive interactions. This suggests that binding regions must be presented in the appropriate context in the yeast two-hybrid assay in order to detect a given interaction, and that small deviations can prevent detection of the interaction. It is likely that this phenomenon contributes to the large number of false-negatives in the yeast two-hybrid assay. Others have also noted that the use of fragments enables interactions to be identified that may be missed when full-length genes were used [15,16]. Similarly, yeast two-hybrid screens of activation domain libraries containing fragments of varying sizes and sequences often yield more positives than screens of the same constructs against arrays of full-length genes [15,17]. However, we must also add a note of caution. Though this approach successfully identified an interaction mediated by a relatively small interaction domain, some interaction domains are much larger. The densely overlapping fragmentation approach is unlikely to identify these interactions because full-length *P. falciparum* genes and large fragments are poorly expressed in yeast. In addition, this method will likely be limited by the same factors that affect other yeast two-hybrid approaches. Some interactions can be identified in one version of yeast two-hybrid assay but not others [18], or require the interacting partners be cloned in a particular orientation (activation domain versus DNA binding domain) or at a particular end of the activation or DNA binding domains [19].

As previously reported, cell free translation in wheat germ extracts efficiently synthesized *P. falciparum* fusion proteins for confirmatory assays. For example, the data shown in Fig. 2 were generated from the equivalent of 0.12 μ l of in vitro translation reaction. Although both confirmation approaches yielded equivalent results, the split-luciferase assay had significant advantages over GST pulldowns. The split-luciferase assay was far easier to implement, required less hands-on time, involved no wash steps, and needed less optimization to find conditions that gave a reasonable signal to noise ratio. Because the assay can be performed in 96-well plates, many pairs can be evaluated simultaneously and multiple replicates can be performed for each pair being tested. Although it was initially envisioned solely as a tool to rapidly validate yeast two-hybrid interactions, future experiments will investigate the potential to use the split-luciferase assay as a screen for novel interactions.

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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.2011.04.005.

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