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In eukaryotes, the high-mobility-group (HMG) nuclear factors are highly conserved throughout evolution and are divided into three families, including HGMB, characterized by an HMG box domain. Some HMGB factors are DNA structure specific and preferentially interact with distorted DNA sequences, trigger DNA bending, and hence facilitate the binding of nucleoprotein complexes that in turn activate or repress transcription. In *Plasmodium falciparum*, two HMGB factors were predicted: PfHMGB1 and PfHMGB2. They are small proteins, under 100 amino acids long, encompassing a characteristic HMG box domain closely related to box B of metazoan factors, which comprises two HMG box domains, A and B, in tandem. Computational analyses supported the conclusion that the *Plasmodium* proteins were genuine architectural HMGB factors, and in vitro analyses performed with both recombinant proteins established that they were able to interact with distorted DNA structures and bend linear DNA with different affinities. These proteins were detected in both asexual- and gametocyte-stage cells in Western blotting experiments and mainly in the parasite nuclei. PfHMGB1 is preferentially expressed in asexual erythrocytic stages and PfHMGB2 in gametocytes, in good correlation with transcript levels of expression. Finally, immunofluorescence studies revealed differential subcellular localizations: both factors were observed in the nucleus of asexual- and sexual-stage cells, and PfHMGB2 was also detected in the cytoplasm of gametocytes. In conclusion, in light of differences in their levels of expression, subcellular localizations, and capacities for binding and bending DNA, these factors are likely to play nonredundant roles in transcriptional regulation of *Plasmodium* development in erythrocytes.

Malaria is the most important parasitic disease in the world, and of the 300 to 500 million cases each year, approximately 2 million people die. Among the four species of malaria parasites infecting humans, *Plasmodium falciparum* causes the highest morbidity and mortality. Global efforts to eradicate malaria have failed, and there is presently no effective vaccine available. Therefore, a greater understanding of parasite biology throughout development is urgently needed in order for novel therapeutic strategies to control malaria to be proposed.

During the erythrocytic life cycle, intense multiplication of parasites takes place, as well as gametocyte differentiation associated with cell cycle arrest. These different developmental pathways require the coordinated and modulated expression of diverse sets of genes, involving transcriptional, epigenetic, and posttranscriptional regulation. Currently, it is commonly accepted that general mechanisms involved in gene regulation in eukaryotes also operate in *P. falciparum* (25, 32, 33). Nevertheless, elucidation of the molecular mechanisms involved in transcriptional regulation in *Plasmodium* is still challenging.

Even if very little is known about the *cis*- and *trans*-regulatory elements of the parasite, *Plasmodium* genes exhibit the bipartite structure of eukaryotic promoters, i.e., a basal promoter regulated by upstream regulatory elements (25) that present some homology with the binding sites of eukaryotic transcription factors (TF). The recent completion of the genome sequence of *P. falciparum* revealed a high proportion of orphan proteins (60% of the open reading frames [ORFs] have no match with any of the annotated sequences listed in the data banks [18]). These data might contribute to the low numbers of recognizable, orthologous TF (11). However, it is reasonable to assume that in *Plasmodium* the interplay between regulatory elements and TF, whose availability (49) presumably modulated throughout parasite development, governs also the level of RNA synthesis.

In eukaryotes, in addition to general TF also annotated in *Plasmodium* (10, 23, 37, 38, 43, 44), the factors involved in transcriptional regulation can be divided into factors interacting either with specific DNA sequences (42) or with DNA structures. The latter include the nonhistone proteins of the high-mobility-group (HMG) superfamily (7, 58, 62), which is divided into three families of proteins in line with their characteristic functional motifs (8): HMGA, which interacts with the AT hook; HMGN, which interacts with the nucleosomes; and HMGB, which encompasses one or several copies of the HMG box DNA binding domain (for a review, see reference 7). HMG proteins are present in all metazoan phyla, plants, and yeast and have also been reported in unicellular parasites, including trypanosomes (15, 45), schistosomes (21), and *Plasmodium* (29). They are quite abundant proteins, one molecule

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for 10 to 15 nucleosomes in vertebrates. It is assumed that the wrapping of DNA by histones and nonhistone proteins, including the HMG proteins, controls the access of the TF to their target sites on nucleosomes (31).

HMGB factors are highly conserved throughout evolution, and their HMG box domain is composed of around 80 amino acids (aa) folded in three α-helices arranged in an L shape (3, 66). In vertebrates, the HMGB proteins generally present two boxes, A and B, and also basic N- and C-terminal extensions and a rather long C-terminal acidic tail (58). Despite their low sequence homology, both boxes (A and B) present a well-conserved L-shaped structure, even though their DNA binding and bending capacities may display some differences (28, 69). In lower eukaryotes, either the basic extension (*Drosophila melanogaster*) or the negatively charged tail (*Saccharomyces cerevisiae*) is missing, in contrast to plant HMGs (60), which possess both extensions, albeit of different lengths. The basic domains appear to play a role in the stabilization of HMGB-mediated DNA bending. In contrast, the role of the acidic tail remains elusive and may be shaped to interact with the positive charges of histones (for a review, see reference 62). Two sub-families of HMGB, with either DNA sequence specificity (SOX, SRY, TCF, MATA) or structure specificity (HMGB per se), have been identified. The latter preferentially interacts with distorted DNA sequences and triggers DNA bending, hence altering the positioning of nucleosomes on the DNA fiber, thereby controlling the level of transcription (31). Finally, a linker histone H1 (27), via its interaction with the DNA linker between two nucleosomes, increases the compactness of the chromatin (53), impairing interactions between DNA and TF and therefore repressing gene transcription (24). In contrast, the HMGB proteins appear to be associated with active chromatin (48), increasing nucleosome sliding and target site accessibility and thereby enhancing transcription.

Lately, these proteins, historically known as nuclear proteins, have been reported to be released from mammalian cells and to act as mediators of the immune response and as potent macrophage-activating factors (for reviews, see references 14, 41, and 46).

In *Plasmodium*, very few TF have been annotated (PlasmoDB and our group [19]) and characterized (6, 20). In *Plasmodium falciparum*, four potential HMG factors have been annotated, including one previously reported for the FCQ27 (29) and FCC1/HN parasite clones. Two of these factors, PfHMGB1 and PfHMGB2, were investigated during the erythrocytic cycle to evaluate their molecular implications in transcription regulation.

MATERIALS AND METHODS

***Plasmodium falciparum* culture.** The 3D7 clone of *P. falciparum* was provided by D. Walliker and was grown in human erythrocytes, as described by Trager and Jensen (61), except that the culture medium contained 0.5% Albumax instead of human serum. Gametocytes were produced from the gametocyte-producing isolate NF54 (provided by W. Eling) as aforementioned, with 10% AB human serum, using the automated culturing system developed by Ponnudurai et al. (52). Parasitemia was monitored on Giemsa-stained blood smears. Thirteen to 15 days after induction of gametocytogenesis, gametocytes were monitored for maturity by observation under a microscope (1 × 100 immersion) of microgamete exflagellation, with the addition of 5 μl of gametocyte culture in a 10-μl drop of human serum.

Antibodies. Primary antibodies used to characterize the PfHMGB1 and PfHMGB2 proteins were obtained after immunization of BALB/c mice with either recombinant protein (50 μg, twice at 2-week intervals). Sera with high levels of

anti-PfHMGB1 and anti-PfHMGB2 activity were collected after the first booster inoculation (day 30) and selected for immunochemical analyses.

Annotation. Before the sequence of the *P. falciparum* genome was completed in 2002, PfHMGB1 was annotated within chromosome 12 by homology to a consensus of the HMG box domains of around 50 disparate eukaryotic sequences. Then, the HMG box domain of PfHMGB1, used as a query against the *Plasmodium* database, allowed the annotation of PfHMGB2 and PfHMGB3 within chromosomes 8 and 12, respectively, while that of PfHMGB2 allowed the annotation of PfHMGB4 within chromosome 13. The presence of relevant HMG box domains in these proteins was checked with MotifScan (16).

Molecular cloning. The Pf*hmgb1* and Pf*hmgb2* ORF sequences were amplified from genomic DNA of the *P. falciparum* 3D7 clone, with forward (5′-GGTGG ATCCATGAAGAATACAGGAAAAGAAG-3′ and 5′-GGTCCCGGGGCCA ATTTAAGCTTTCATTTTC-3′) and backward (5′-ATTGGATCCATGGCTTC AAAATCTCAAAA-3′ and 5′-ATTGGTACCTTATTCTTGATTTTTCTTTC-3′) primers, respectively, using PCR conditions with an elongation temperature of 60°C, as described previously (57). Fragments of 294 bp and 300 bp, corresponding to the complete Pf*hmgb1* and Pf*hmgb2* ORF sequences, were cloned directly into the pGEM-T Easy vector (Promega) and pCR II-Topo (Invitrogen), respectively, and then sequenced with the ABI Prism kit (Perkin Elmer).

Northern blot analysis. Total RNA was purified from isolated parasites with TRIzol (Invitrogen), and the integrity of the RNA preparation was monitored by ethidium bromide staining on an agarose gel and analysis with an Agilent bioanalyzer. The Pf*hmgb1* and Pf*hmgb2* transcripts were characterized from 20 μg of 3D7 total RNA by Northern blotting, according to the Ultrahyb protocol of Ambion, with a 65°C hybridization temperature. Antisense α-^{32}P riboprobes were prepared as previously described (50) from the pGEMT-*pfhmgb1* and pCRII Topo-*pfhmgb2* vectors by using T7 and SP6 RNA polymerase (Promega), respectively.

Expression and purification of recombinant proteins. The pQE30 vector (QIAGEN) was used to express the recombinant proteins (rePfHMGB1 and rePfHMGB2) in *Escherichia coli* as His$_6$-tagged proteins with BamHI-XmaI- and BamHI-KpnI-digested inserts from the above-mentioned vectors, respectively. The bacterial strain SG 13009, harboring the Pf*hmgb1* or Pf*hmgb2* expression construct, was grown at 37°C in 200 ml of 2YT medium containing 100 μg/ml ampicillin as well as 50 μg/ml kanamycin. Expression was induced with 0.1 mM IPTG (isopropyl-β-ᴅ-thiogalactopyranoside) for 3 h at 37°C, and collected cells were solubilized in sonication buffer S (25 mM Tris-HCl [pH 8], 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol, 0.5% Triton X-100), 1 ml per mg of dry pellet, in the presence of lysozyme and a 1/25 final dilution of a protease inhibitor cocktail tablet (Roche). Purification of His-PfHMGB proteins was performed essentially as previously described with Ni-nitrilotriacetic acid agarose beads (QIAGEN) (34). After three washes with buffer S supplemented with 20 mM imidazole, bound proteins were eluted with either 250 mM or 50 mM imidazole in 20 mM Tris-HCl (pH 8)–300 mM NaCl for rePfHMGB1 or rePfHMGB2, respectively.

EMSA with synthetic four-way DNA junctions. The partially complementary oligonucleotides 1 to 4, previously described (5, 67), were used to create four-way DNA junctions (4H), as well as 3H and 2H. In addition, the radiolabeled leg 1 containing cruciform 4H was fractionated and eluted from a 5% polyacrylamide gel in 0.5× Tris-borate-EDTA (TBE) buffer. Electrophoretic mobility shift assays (EMSA) were performed by incubating labeled 4H with increasing amounts (0 to 25 μM) of rePfHMGB1 or rePfHMGB2 in a 10-μl final volume for 20 min at room temperature. This dose-response experiment was carried out to determine the amounts of protein necessary to create a major 4H-PfHMGB complex. In the competition assay, 100- and 500-fold molar excesses of cold complete 4H or incomplete 3H and 2H were added to the reaction for an additional 20 min. Samples were run on a 6.5% polyacrylamide gel in 0.5× TBE buffer at 120 V. The vacuum-dried gels were autoradiographed with intensifying screens at −80°C overnight.

Ligase-mediated circularization assay. The circularization assay was based on existing protocols (9, 67). Linear DNA fragments of 123 bp were γ-^{32}P 5′ end labeled and preincubated with increasing concentrations (0.25 to 100 μM) of rePfHMGB1 or rePfHMGB2 for 20 min at room temperature in 1× DNA ligase buffer (New England Biolabs [NEB]) in a final volume of 10 μl. DNA circularization was generated with T4 DNA ligase (NEB), and linear DNA was subsequently digested by exonuclease III (NEB). Samples were treated with proteinase K (Invitrogen) and run on a 6.5% polyacrylamide gel in 0.5× TBE buffer at 120 V. The gels were vacuum dried and autoradiographed.

Preparation of parasite NE, CE, and total extracts and Western blot analysis. Nuclear extracts (NE), cytoplasmic extracts (CE), and total lysates were prepared from 50 ml of red blood cells at 10 to 12% parasitemia infected with 3D7 asexual-stage cells, as described by Osta et al. (49). Proteins (12 μg) were run on

12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to Western blotting experiments (see Fig. 4b) after transfer onto polyvinylidene difluoride membranes (Bio-Rad). For sexual-stage analysis, 2 ml of NF54 culture was harvested on day 13 after induction of gametocytogenesis, with 13.8% parasitemia including 55% mature gametocytes (stage V). After erythrocytic lysis in phosphate-buffered saline–0.15% saponin, gametocytes were collected and resuspended in 40 μl of Laemmli buffer. Half of this sample was fractionated for detection of either protein, PfHMGB1 or PfHMGB2. A similar amount of asexual-stage culture was run in parallel (see Fig. 4c). The blots were probed with a 1:2,000 dilution of anti-PfHMGB1 or anti-PfHMGB2 serum, followed by incubation with a peroxidase-conjugated anti-mouse immunoglobulin G (IgG) antibody (Sigma), and revealed by chemiluminescence (Perkin Elmer) or the Supersignal West Femto kit (Pierce). Negative controls were performed with preimmune sera and positive controls with the recombinant proteins. Finally, an anti-HSP70 serum (55) was used as a positive control for CE (see Fig. 4b) and for normalization of protein loading between asexual/sexual stages (QuantiScan Biosoft 2.1).

Immunofluorescence assay. For localization of PfHMGB1 and PfHMGB2 in *P. falciparum*, asexual and sexual stages obtained as described previously were washed twice in RPMI, fixed in 2% paraformaldehyde for 20 min at room temperature, and laid on poly-L-lysine-coated multiwell glass slides. Endogenous fluorescence was quenched with 75 mM NH_4Cl for 10 min. The parasites were permeabilized with 0.5% Triton X-100 for 3 min, blocked with 0.5% bovine serum albumin in phosphate-buffered saline for 1 h, and then incubated with a 1:200 dilution of primary antibodies (anti-PfHMGB1, anti-PfHMGB2, anti-HSP70) for 1 h at room temperature, followed by incubation with a fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (Sigma) and DAPI (4′,6′-diamidino-2-phenylindole) for 1 h before examination by fluorescence microscopy using a Leica DM RD fluorescent microscope equipped with UV and fluorescein filters. The time of exposure was selected in relation to the intensity of the immunofluorescence obtained with each antibody. Images were acquired with Lucia 4.7 and merged with Adobe Photoshop.

Nucleotide sequence accession numbers. The nucleotide sequences of the genes encoding the following proteins have been assigned the corresponding PlasmoDB accession numbers: PfHMGB1, PFL0145c; PfHMGB2, MAL8P1.72; PfHMGB3, PFL0290w; PfHMGB4, MAL13P1.290.

RESULTS

Several predicted factors in *Plasmodium* belong to the HMGB family. Four putative HMG proteins in *P. falciparum* were predicted by sequence homology, as described in Materials and Methods. The proteins appeared to belong to the HMGB family and were named PfHMGB1 to PfHMGB4. The first two, PfHMGB1 and PfHMGB2, are small proteins, under 100 aa in length, while PfHMGB4 has been predicted to encode a 160-aa-long protein. All three encompass only one HMG box domain. In contrast, PfHMGB3 is a larger protein (2,284 aa), with two HMG box domains and several additional putative functional motifs, including one Myb domain (2). The two small proteins, PfHMGB1 and PfHMGB2, are presented herein. It is worthy of note that in contrast to most eukaryotic HMGBs, displaying N- and C-terminal extensions of diverse lengths (58) and reported to bear functional roles, the two *Plasmodium* factors have only a short basic extension upstream of the HMG box domain and no acidic C-terminal tail.

Soullier et al. (56) revealed by phylogenetic analysis that the HMGB factors can be separated into two clearly defined subgroups: (i) the SOX/SRY/MATA/TCF family, whose members are able to bind specific linear DNA sequences, and (ii) the HMGB/UBF family, whose members interact with high affinity to distorted DNA structures. After the addition of *Plasmodium* and *Babesia bovis* sequences, the same analysis was performed several times with different random number seeds, and we were able to assign PfHMGB1 and PfHMGB2 to the subgroup of HMGB proteins characterized by DNA

structure specificity (see supplemental material S1) (17, 59). This finding was strengthened by the alignment shown in Fig. 1, performed with several eukaryotic HMG box domains and two sets of complete HMGB1 and HMGB2 sequences issued from diverse *Plasmodium* species. The HMGB of *Plasmodium* possessed two of the three determinants reported to determine the structural DNA specificity (47), that is, the presence in positions 10 and 32 (according to the residue numbering of *Drosophila* HMG-D) of a serine and a hydrophobic residue, respectively. Therefore, we assigned the *Plasmodium* factors to the architectural HMGB family. Finally, a phylogenetic tree was issued from the HMG box domains of PfHMGB1 and PfHMGB2 and those of various metazoan proteins containing two boxes, A and B, in tandem (see supplemental material S2). This analysis, performed several times with different random number seeds, revealed that the HMG box sequences of both *Plasmodium* proteins are more similar to box B than to box A of proteins with two HMG box domains.

The three-dimensional structure of the two *Plasmodium* factors was modeled by homology using as template the structure of box B of the Chinese hamster HMG1 protein (PDB file 1hsn [54]). Four α-helices, called 1, 1′, 2, and 3 (underlined residues in Fig. 1 corresponding to the three α-helices, I, II, and III, stated at the top of Fig. 1 for *D. melanogaster* HMG-D), were predicted to fold in an L shape (see supplemental material S3) (12, 30) in the PfHMGB1 sequence from His 19 to Tyr 90 and in the PfHMGB2 sequence from Ala 23 to Gln 98.

Therefore, all of these computational analyses agreed in suggesting that the two PfHMGB proteins were genuine factors of the HMGB family and may therefore behave as potential architectural factors.

PfHMGB1 and PfHMGB2 interact with 4H. Several sets of in vitro assays were used to validate the computational identification. After the genes were cloned, expression of rePfHMGB1 and rePfHMGB2 was carried out in *Escherichia coli* and His proteins were purified as described in Materials and Methods.

First, increasing amounts of both recombinant rePfHMGB1 and rePfHMGB2 (up to 25 μM) were incubated with radiolabeled complete 4H to analyze the formation of 4H-rePfHMGB1 and 4H-rePfHMGB2 complexes by EMSA. When 3 μM rePfHMGB1 (Fig. 2a) or 0.6 μM rePfHMGB2 (Fig. 2b) was added to the reaction, the 4H labeled cruciform became incorporated into a major 4H-PfHMGB1 or 4H-PfHMGB2 retarded band, respectively. These amounts of recombinant factors were used for the subsequent EMSA experiments.

Second, the binding specificities of the 4H-PfHMGB1 and 4H-PfHMGB2 complexes were analyzed by competition experiments, that is, by adding a 100- or 500-fold molar excess of either complete (4H) or incomplete (3H and 2H) cold DNA junctions, after DNA-protein complex formation. A 500-fold excess of the integral 4H structure abolished the interaction of either protein, whereas a 100-fold molar excess shifted the 4H-PfHMGB2 interaction completely but the 4H-PfHMGB1 interaction only weakly (Fig. 2c and d). In addition, the competition with cold 3H and 2H was ineffective upon analysis of PfHMGB1, in contrast to the substantial competition observed in the presence of PfHMGB2. Thus, binding of PfHMGB1 to 4H required an intact crossover-containing structure. Alto-
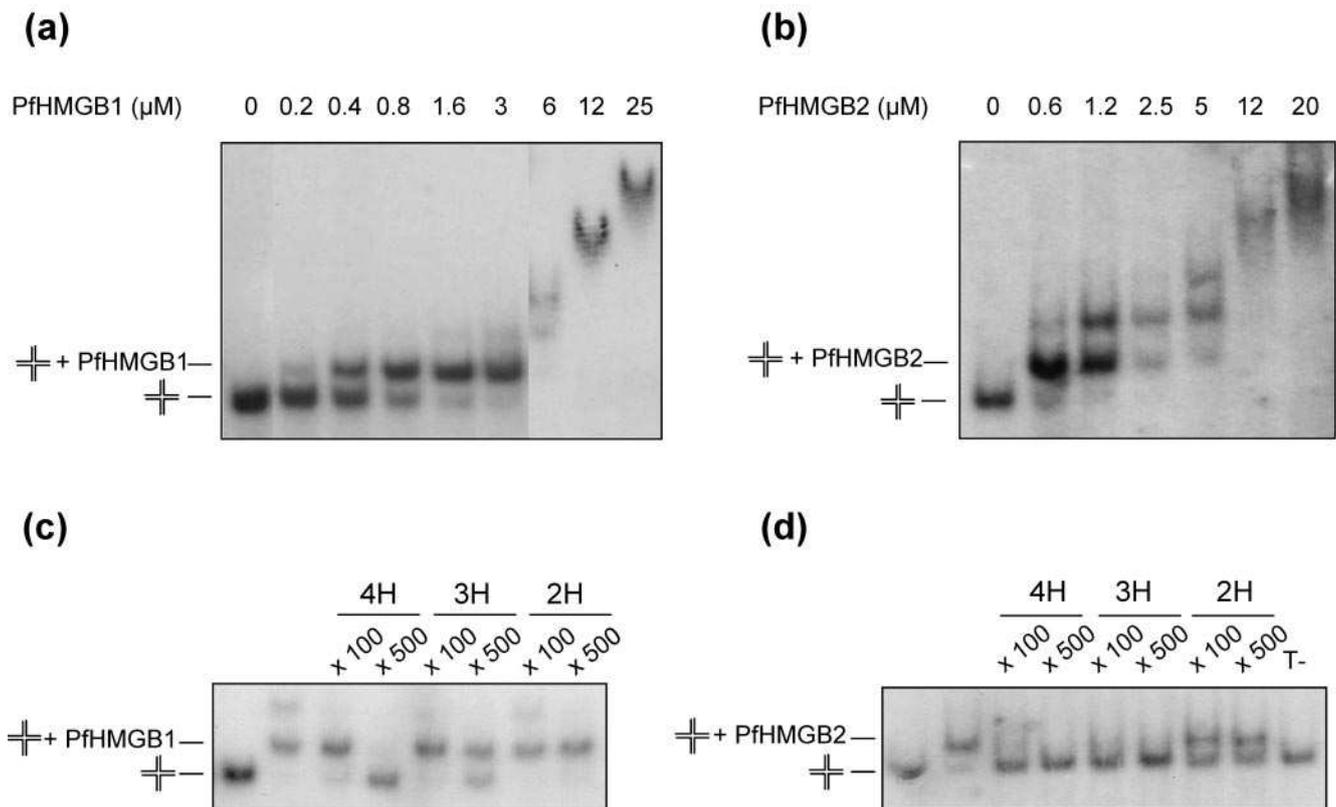
I   II   III

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Dm.HMG-D                                        msdkPKRPISAYMLWLNSARESIKRENP--GIKVTEVAKRGGELWRAM--KDKSEWEAKAAKAKDDYDRAVKEFEangs............
Rn.HMG1_A                                       mgkgdPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIppkg............
Hs.HMG1_A                                       mgkgdPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSKKCSERWKTMSAKEKGKFEDMAKADKARYEREMKTYIppkg............
Hs.HMG2_A                                       mgkgdPNKPRGKMSSYAFFVQTCREEHKKKHPDSSVNFAEFSKKCSERWKTMSAKEKSKFEDMAKSDKARYDREMKNYVppkg............
Zm.MNB1b          ...................kagkdpnkPKRAFSAFFVFMEEFRKEFKEKNPK-NKSVAAVGKAAGDRWKSLSESDKAPYVAKANKLKLEYNKAIAAYNkges............
Os.HMGB1          ...................kagkdpnkPKRAFSAFFVFMEEFRKEFKEKNPK-NKSVAAVGKAAGDRWKSLTEADKAPYVAKANKLKAEYNKAIAAYNkges............
Gm.HMG1           ...................kaakdpnkPKRPFSAFFVFMEEFRKVFNKEHPE-NKAVAAVGKAAGAKWKTMSDAEKAPYVAKSEKRKVEYKNMRAYNkkqa............
Bb.NHP1           ...................kakkdpnaPKRAISSYMFFAKEKRVEIIAENPEIAKIDVAAIGKMIGAAWNALSDEEKKPYERMSDEDRVRYEREKAEYAqrkv............
Rn.HMG1_B         ..................k-fkdpnaPKRPFSAFFLFCSEYRPKIKGEHP--GLSIGDVAKKLGEMWNNTAADDKQPYEKKAAKLKEKYEKDIAAYRakgk............
Hs.HMG1_B         ..................k-fkdpnaPKRPFSAFFLFCSEYRPKIKGEHP--GLSIGDVAKKLGEMWNNTAADDKQPYEKKAAKLKEKYEKDIAAYRakgk............
Hs.HMG2_B         ..................k-kkdpnaPKRPFSAFFLFCSEHRPKIKSEHP--GLSIGDTAKKLGEMWSEQSAKDKQPYEQKAAKLKEKYEKDIAAYRakgk............
Sc.NHP6A                                mvtprepkkrttrkkkdpnaPKRAISAYMFFANENRDIVRSENPDIT--FGQVGKKLGEKWKALTPEEKQPYEAKAQADKKRYESEKELYNatla

Pv.HMGB1     mggnkshnnrvrsrhparkmdpmkkfngmknmggkev--rkrrknkkdphaPKRSISAYMFFAKEKRAEIISRDPDLSKIVATVGKMIGEAWNKLDEREKAPYEKKAQEDKLRYEREKVEYAktkmka
Py.HMGB1          mdgmkkfkdmk-mggkev--kxrrknxkkdphaPKRSISAYMFFAKEKRAEIITRDPSLSKIVATVGKMIGEAWNKLDEREKAPYEKKAQEDKIRYEKEKMEYAknkmk
Pb.HMGB1          mdgmkkfkdmk-mggkev--kxrrknxkkdphaPKRSISAYMFFAKEKRAEIITRDPSLSKIVATVGKMIGEAWNKLDEREKAPYEKKAQEDKIRYEKEKMEYAksknk
Pk.HMGB1          mdpmkkfngmknmggkev--rkrrknkkdphaPKRSISAYMFFAKEKRAEIISRDPDLSKIVATVGKMIGEAWNKLDEREKAPYEKKAQEDKVRYEREKVEYAktkmka
Pf.HMGB1               mkntg-kev--rkrrknkkdphaPKRSISAYMFFAKEKRAEIISKQPELSKIVATVGKMIGEAWNKLGEKEKAPFEKKAQEDKLRYEKEKAEYAnmkmka

Pf.HMGB2          masksqkkvlkkqnkkkkkdplaPKRAISAYMFYVKDKRLEIIKEKPELAKDVQVGKLIGEAWQLSPAQKAPYEKKAQLDKVRYSKEIIEYRknqe
Pk.HMGB2          maaksqkkvlrkqnkrkkkdplaPKRAISAYMFYVKDKRLELIKEKPELARNVAQVGKLVGEAWKLSAAQKTPYEKKAQLDKVRYSKEIIEYRtkne
Pb.HMGB2          matktqkkvikkqmkkkkkdplaPKRAISAYMFYVKDKRLEIIQFRPELAKEVAQVGKLIGEAWGQLTPAQKAPYEKKAELDKVRYSKEIIEYRtke
Pv.HMGB2     mctwgqsnkmaaksqkkvlkkqnkrkkkdplaPKRAISAYMFYVKDKRLELIKERPELAKDVAQVGKLVGEAWQLSAAQKTPYEKKAQLDKVRYSKEIIEYRtkkemkkkkkakslgr
Py.HMGB2     myiyyilssvlymssyifyefvskqidkmatktqkkvikkqnkkkkkdplaPKRAISAYMFYVKDKRLEEIQERPELAKEVAQVGKLIGEAWQLTPAQKAPYEKKAELDKVRYSKEIIEYRtke
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FIG. 1. Multiple alignment of HMG box domains of several eukaryotic HMG proteins and complete sequences of *Saccharomyces* NHP6A and HMGB1 and HMGB2 of diverse *Plasmodium* species. Dashes and dots represent gaps and missing sequences, respectively. Uppercase letters represent HMG box domains identified by the program MotifScan (16). Identities shared by all HMGB sequences presented herein are indicated in bold blue print, by all *Plasmodium* sequences in green, by all *Plasmodium* HMGB1 sequences in red, and by all *Plasmodium* HMGB2 sequences in pink. The boxed amino acids are two crucial determinants that differ between the sequence-specific and the structure-specific HMG box domains: here, a serine and a hydrophobic residue, as found in all non-sequence-specific HMG proteins, whereas all sequence-specific HMG proteins present an asparagine and a hydrophilic residue at these positions. I, II, and III represent the three α-helices of the *D. melanogaster* HMG-D structure (PDB file 1qrv [47]), and asterisks indicate the two residues of *D. melanogaster* HMG-D that intercalate in the DNA minor groove. Underlined residues in PfHMGB1 and PfHMGB2 represent the four α-helices modeled by homology with box B of the Chinese hamster HMG1 protein, used as a template (PDB file 1hsn [54]). Abbreviations are as follows: Bb, *Babesia bovis*; Dm, *Drosophila melanogaster*; Gm, *Glycine max*; Os, *Oryza sativa*; Pb, *Plasmodium berghei*; Pf, *Plasmodium falciparum*; Pk, *Plasmodium knowlesi*; Pv, *Plasmodium vivax*; Py, *Plasmodium yoelii*; Rn, *Rattus norvegicus*; Sc, *Saccharomyces cerevisiae*; Zm, *Zea mays*.

(a)

(b)

(c)

(d)

FIG. 2. EMSA interaction between cruciform DNA and either rePfHMGB1 (a) or rePfHMGB2 (b). Increasing concentrations (0 to 25 μM) of rePfHMGB proteins were incubated with radiolabeled 4H. Competition EMSA experiments were performed between the rePfHMGB1-4H (c) or rePfHMGB2-4H (d) complex and various DNA competitors. After incubation of the rePfHMGB proteins with labeled 4H, either cold incomplete junctions (3H and 2H) or complete 4H were added to the reaction at 100- and 500-fold molar excesses, as indicated above each lane. ⊹, cruciform DNA. The first lanes correspond to cruciform DNA migration without protein interaction (a to d), and the second lanes correspond to a cruciform DNA-rePfHMGB complex retarded band without competition (c and d).

gether, these results indicated that the two recombinant proteins were able to bind distorted DNA in vitro, even though the nature of their interaction with 4H was quite different, being more efficient and specific for PfHMGB1 than for PfHMGB2.

PfHMGB1 and PfHMGB2 induce DNA bending. We compared the efficiencies of increasing concentrations of rePfHMGB1 and rePfHMGB2 to bend and in turn promote T4 DNA ligase-mediated circularization of a labeled synthetic linear DNA fragment. Indeed, when ligase was added to the labeled fragment of around 125 bp, several bands appeared, including a circular DNA form resistant to exonuclease III (Fig. 3a and b). In the presence of exonuclease III alone, which digests only linear DNA molecules, a marked decrease in all labeled bands was observed, showing that in the absence of PfHMGB proteins, only small amounts of minicircles, if any, were produced. In contrast, in the presence of ligase and increasing amounts of PfHMGB proteins, the quantity of minicircles was quite increased, suggesting that both proteins were capable of enhancing DNA flexibility and hence DNA circularization. The capacity for DNA bending is thus an intrinsic property of PfHMGB1 and PfHMGB2. Nevertheless, rePfHMGB1 once again showed greater efficacy (Fig. 3a), since it started to promote circularization at 0.25 μM, at a concentration 10-fold lower than that of rePfHMGB2 (3 μM), and the maximum

signal was reached with 1 μM compared with 50 μM rePfHMGB2. Moreover, the signal observed with 50 to 100 μM PfHMGB2 (Fig. 3b) was far weaker than that observed with 2 μM PfHMGB1 (Fig. 3a).

Pf*hmgb1* and Pf*hmgb2* transcripts are expressed during the *P. falciparum* erythrocytic cycle. In order to determine the presence of Pf*hmgb1* and Pf*hmgb2* RNA and to characterize them molecularly, we performed a Northern blotting analysis of total RNA prepared from infected red blood cells (Fig. 4a). The lengths of Pf*hmgb1* and Pf*hmgb2* mRNA were estimated at 1.3 and 1.1 kb, respectively. The integrity and quality of total RNA extracts of 3D7 were verified after ethidium bromide staining of the gel. As already described for many other *Plasmodium* messengers, the 5′ and 3′ untranslated regions of both transcripts are quite long, as the coding regions comprise fewer than 300 nucleotides.

PfHMGB1 and PfHMGB2 proteins are present in the *P. falciparum* nucleus. Localization of PfHMGB1 and PfHMGB2 was analyzed by Western blotting analysis using CE and NE of *P. falciparum* asexual stages and specific antisera raised against each recombinant protein. The NE and CE prepared from 3D7 parasites (12 μg), as well as the recombinant proteins rePfHMGB1 and rePfHMGB2 (50 ng), were fractionated by SDS-PAGE, and after transfer, the membranes were developed
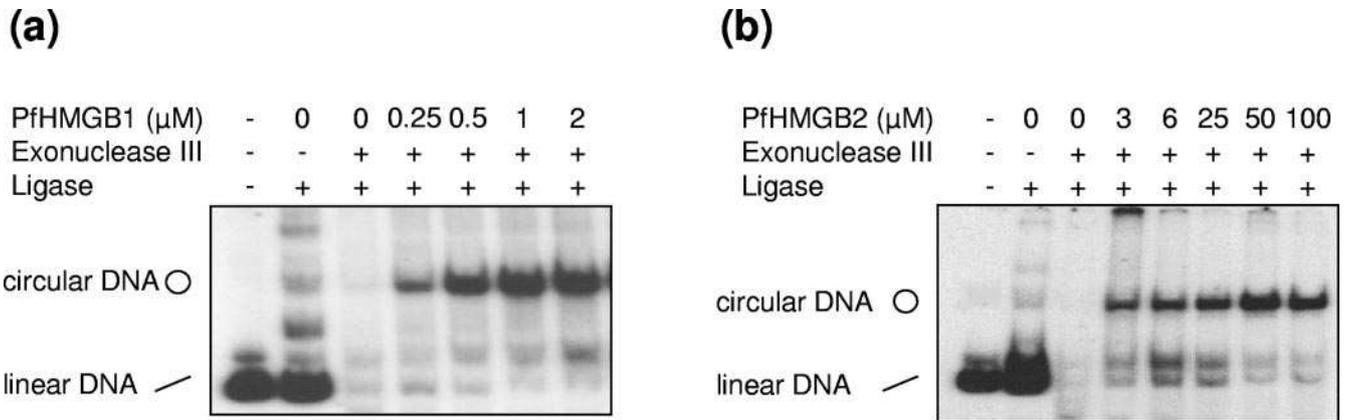
FIG. 3. DNA bending and ligase-mediated circularization assay with either rePfHMGB1 (a) or rePfHMGB2 (b). The γ-^{32}P 5' end-labeled 123-bp DNA fragment was preincubated with increasing amounts of rePfHMGB1 (0 to 2 μM) or rePfHMGB2 (0 to 100 μM), followed by ligation with T4 DNA ligase. The ligation products were subjected to electrophoresis after exonuclease III treatment. T4 DNA ligase was added to all samples except that loaded in the first lane. All samples were treated with exonuclease III except samples of the first two lanes. The migration positions of 123-bp linear and 123-bp minicircular DNAs are indicated at left.

with the specific antisera (Fig. 4b). The specificities of the two antibodies were verified. No cross-reaction was observed, as His-PfHMGB1 and His-PfHMGB2 were recognized only by their respective antisera (Fig. 4b, lanes 4 to 5 and lanes 8 to 9 for His-PfHMGB1 and His-PfHMGB2, respectively). The control experiments performed with the two preimmune sera gave no signal (data not shown). Both PfHMGB1 and PfHMGB2

were clearly detected in the NE (lanes 3 and 7), whereas same protein loading of CE does not give any detectable signal (lanes 2 and 6). The quality of the CE preparations was controlled by detection of the HSP protein (70 kDa) (lane l). The apparent molecular mass of both PfHMGB proteins in the nuclear extracts, around 12 kDa, was in good agreement with the theoretical molecular masses of 11.3 kDa and 11.5 kDa for
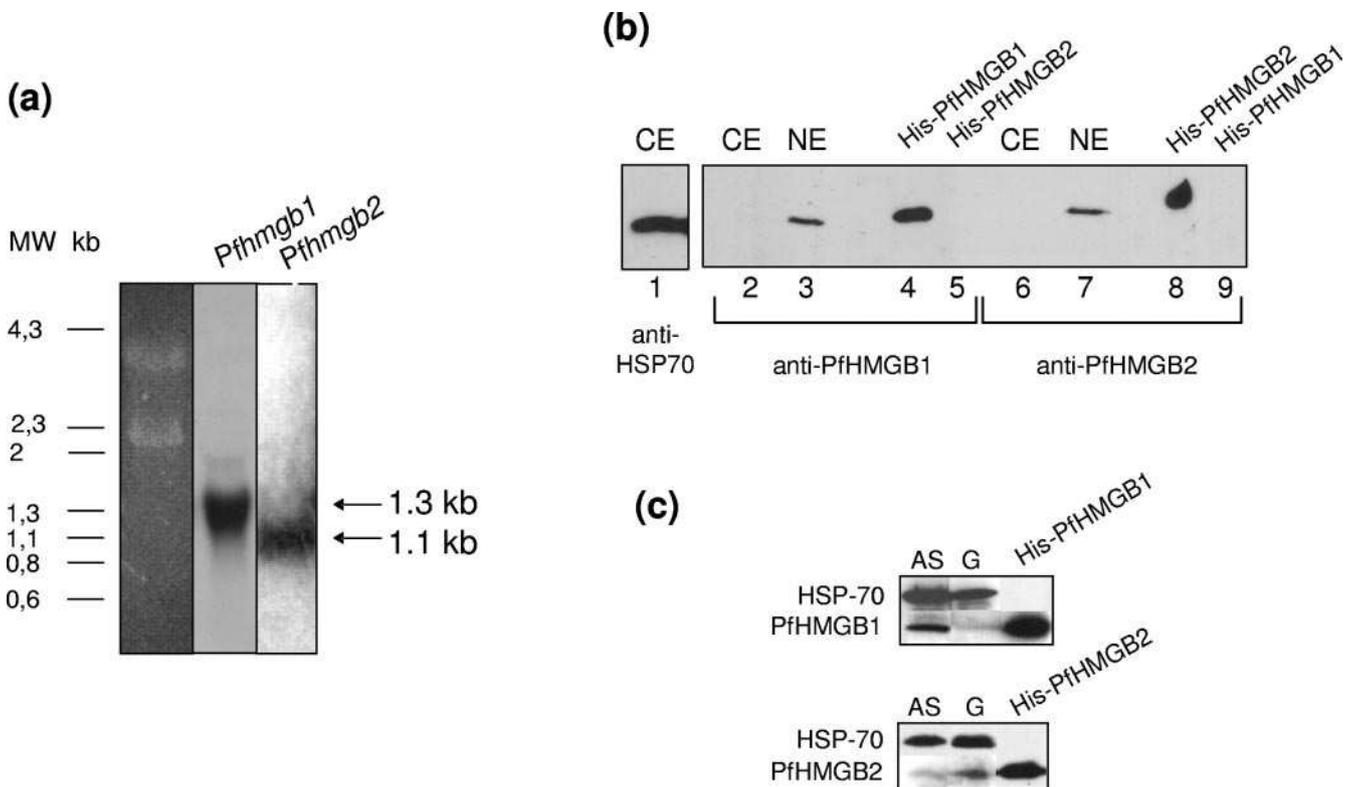
FIG. 4. Characterization of Pf*hmgb1* and Pf*hmgb2* transcripts by Northern blotting (a) and of their corresponding proteins, His-PfHMGB1 and His-PfHMGB2, by Western blotting (b). By SDS-PAGE, the apparent molecular mass of PfHMGB factors was approximately 12 kDa, whereas that of HSP70 was approximately 70 kDa. (c) Expression of PfHMGB1 and PfHMGB2 in total lysates prepared from asexual- versus gametocyte-enriched cultures. HSP70 protein expression was used for normalization of sample loading. AS, asexual stages; G, gametocytes.

PfHMGB1 and PfHMGB2, respectively (lanes 3 and 7), slightly smaller than the recombinant His proteins (12.7 kDa and 12.9 kDa, respectively).

Localization of the factors within the nucleus was confirmed by immunofluorescence analysis of unsynchronized parasite cultures containing all asexual stages of *P. falciparum*. Control experiments carried out in the presence of either preimmune sera or the secondary antibody alone showed no signal (data not shown), in contrast to the labeling obtained with both anti-PfHMGB sera coupled with nucleus-specific DAPI staining (Fig. 5). The two proteins appeared to be present mainly in the parasite nuclei, as shown by the merge of DAPI (lanes a and b), and, in contrast to the HSP70 signal, also readily detectable in the cytoplasm, as indicated by the red fluorescence observed in the superposition insert (lane c).

PfHMGB1 and PfHMGB2 factors are expressed differentially in the asexual and gametocyte stages. We compared the expression levels of both factors within total lysates from mixed asexual versus gametocyte cultures by means of Western blotting experiments (Fig. 4c). Each factor was evaluated as detailed in Materials and Methods via HSP70 expression. The level of PfHMGB1 expression was clearly lower in gametocytes than in mixed asexual stages, in contrast to PfHMGB2, whose expression was higher in gametocytes. Indeed, after densitometric quantification of this representative experiment via HSP70 protein normalization (QuantiScan Biosoft 2.1), the normalized values of protein expression were 71%/29% for PfHMGB1 and 27%/73% for PfHMGB2, respectively, when asexual and gametocyte cultures were compared.

We also compared the localizations of both factors in asexual (red immunofluorescence) and gametocyte (green immunofluorescence) stages. As already mentioned, the two PfHMGB factors (Fig. 5, lanes a and b) appeared to be located mainly in the nucleus of the asexual stages (rings, trophozoites, and schizonts), whereas the HSP70 protein (lane c) was also found in the parasite cytoplasm. Surprisingly, in addition to its nuclear localization, PfHMGB2 could also be readily detected within the cytoplasm of different stages (IV and V) of gametocytes (lanes e), as also observed for the HSP70 protein (lane f), whereas PfHMGB1 was associated mainly with the nucleus of gametocytes, as in asexual parasites (Fig. 5, lanes d and a).

DISCUSSION

The two *Plasmodium* HMG proteins belong to the HMGB subfamily and comprise only one HMG box domain, like proteins of plants and several proteins of yeast and *Drosophila*. The parasite HMG box domains are quite similar to those of all eukaryotic organisms and show characteristic residues that are important for DNA binding and bending, as shown for the *Drosophila* HMG-D protein by Murphy and colleagues (47). The presence of Ser-10 and Val-32 (boxed residues in Fig. 1, according to the numbering of HMG-D) allowed us to assign the *Plasmodium* factors to the architectural HMGB family, as also shown by the phylogenetic analysis performed with 159 HMG box domains (see supplemental material S1). Ser-10 forms water-mediated hydrogen bonds with DNA. The hydrophobic residues Val-32 and Met-13 (Fig. 1, residues with asterisks) partially intercalate between two base pairs, introducing two successive kinks into the bound DNA that enhance the

more uniform bend associated with the widening of the minor groove. In addition to the HMG box domain, the parasite proteins exhibit a basic extension N terminal to the HMG box domain and apparently no acidic C-terminal tail, as also observed for the yeast NHP6A protein. The HMG box domains bind the DNA minor groove, and in the NHP6A and HMG-D proteins, the basic extension binds in the compressed major groove on the face of the helix opposite the widened minor groove (1, 13), so as to stabilize the HMG box domain-induced bending (39) and consequently facilitate circularization (22, 68).

Since the two *Plasmodium* HMGB factors exhibited only one HMG box domain, we asked whether the *Plasmodium* box was more similar to box A or box B of the metazoan HMGB that encompasses two HMG box domains in tandem. All analyses converge to the same conclusion: the *Plasmodium* HMG box domain more closely resembles box B. When the phylogenetic analysis (see supplemental material S2) was performed with the HMG box domains of PfHMGB1 and PfHMGB2 and of various proteins containing box A and box B, the *Plasmodium* factors clearly clustered with all B boxes. For the human HMG1, it was reported that structure-specific binding to the four-way DNA junction was mediated by the A domain (65) and that box B, flanked by the basic region, displayed a marked DNA recognition activity (69). Hence, the short N-terminal basic domain of the two *Plasmodium* nuclear factors might govern their interaction with distorted DNA and subsequent DNA bending.

In addition, box B of the HMGB of vertebrates was reported to behave as a potent proinflammatory cytokine (64). The tumor necrosis factor (TNF)-stimulating activity was mapped to the KDPNAPKRPPSAFFLFCSEY sequence, corresponding to the first 20 aa of box B in human HMG1 factor, according to the numbering of Li et al. (36). In *Plasmodium*, a domain sharing 75% and 70% identical or strongly similar residues with the TNF-stimulating domain of the human factor was found at the N-terminal position of the HMG box domains of PfHMGB1 and PfHMGB2, respectively. Presently, experiments are under way to analyze whether the two *Plasmodium* factors exhibit TNF-stimulating function.

An automatic three-dimensional structural prediction was performed, and for both *Plasmodium* factors, four α-helices, called 1, 1′, 2, and 3, were predicted (underlined residues of PfHMGB1 and PfHMGB2 of Fig. 1), folding in an L shape in the HMG box domain. HMG box domains have actually exhibited only three α-helices (66), but even if four α-helices were predicted in the two parasite proteins, their positions would be in good agreement with those of *Drosophila melanogaster* HMG-D (PDB file 1qrv [47]), which are indicated (I, II, and III) at the top of Fig. 1. In addition, the sample of the reference template used for nuclear magnetic resonance contained a molecule of β-mercaptoethanol attached to the single cysteine of the protein. The molecule of β-mercaptoethanol, which reduces the affinity of the HMG box domain for the four-way DNA junction, also disrupted the usual first helix (see supplemental material S3). For that reason, helices 1 and 1′ could be regarded as a single, kinked helix.

These computational analyses were concordant in suggesting that the *Plasmodium* proteins were genuine architectural HMGB factors close to box B in being able to bind and bend DNA. In vitro analyses performed with both recombinant pro-
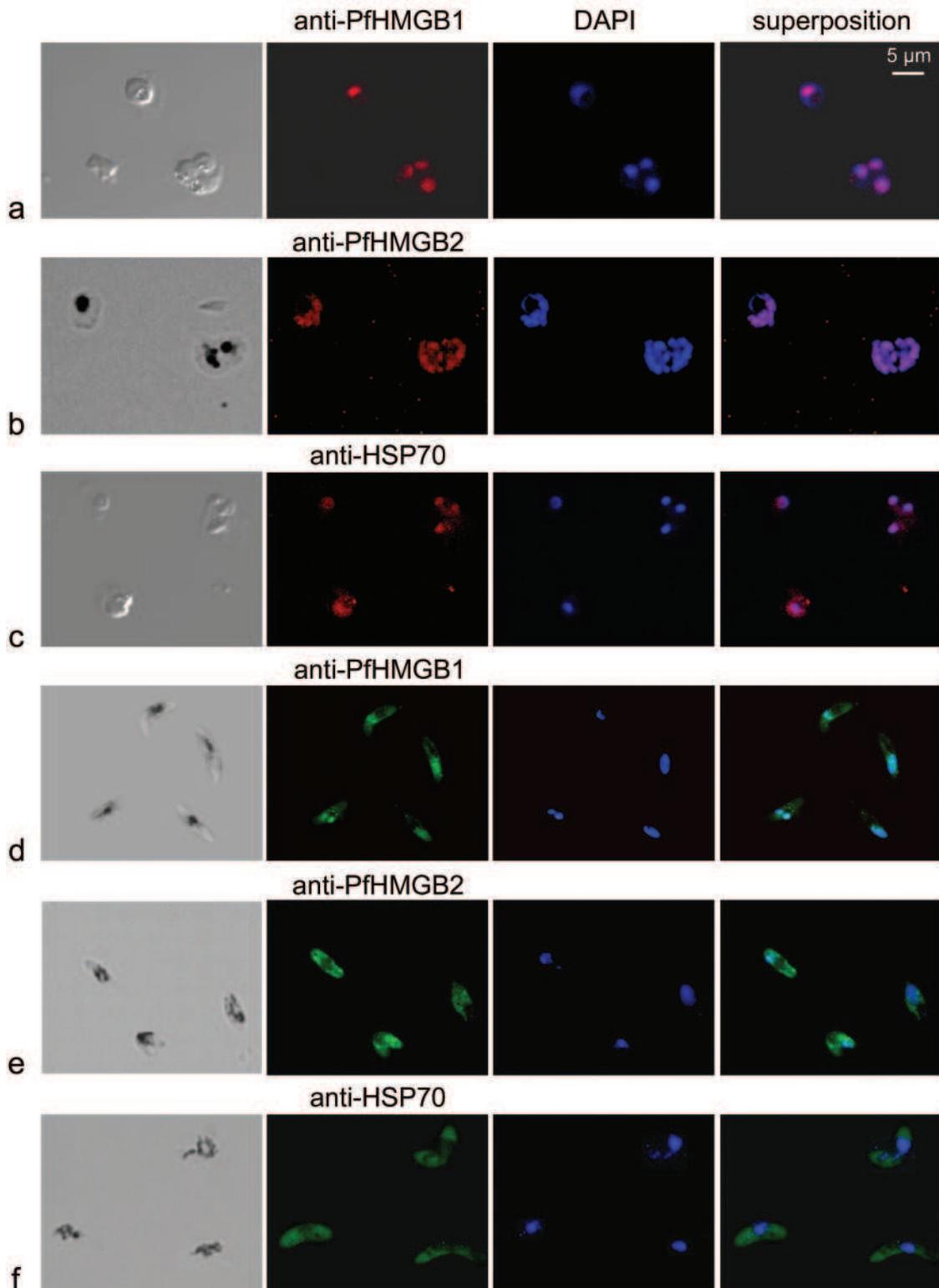
FIG. 5. Immunofluorescence localization of PfHMGB1 and PfHMGB2 in asexual (a, b, and c) and sexual (d, e, and f) stages of *Plasmodium* erythrocytic development. Paraformaldehyde-fixed parasites were labeled with mouse anti-PfHMGB1 and anti-PfHMGB2 antibodies (1:200) and FITC-conjugated anti-mouse IgG (1:100); DNA was stained with DAPI (1:100). Merged fluorescent signals are shown in the "superposition" column. Cells were visualized by phase-contrast (a and c) or transmission (b, d, e, and f) microscopy. Panels: a, trophozoites; b, trophozoite and schizont; c, trophozoites; d to f, gametocytes. Anti-PfHMGB and anti-HSP70 fluorescence is red for panels a to c and green for panels d to f.

teins established that they were indeed able to interact with distorted DNA structures (Fig. 2) and bend linear DNA (Fig. 3), leading to the validation of the computational data (Fig. 1 and supplemental materials S1, S2, and S3). In contrast, EMSA performed with labeled linear DNA binding sites reported to interact specifically with members of the HMGB subfamily comprising the usual TF SOX and SRY (63) gave no detectable retarded complexes (data not shown).

Therefore, it can reasonably be assumed that these architectural HMGB factors might play a role in the remodeling of chromatin. In eukaryotes, one proposed mechanism is that the HMGB nuclear factors might change the nucleosome structure and relax the wrapped DNA so as to enhance the accessibility of the remodeling complexes to chromatin and facilitate interaction of TF with their binding sites (for a review, see reference 62). It has also been observed that the interplay between these factors and the linker histone H1 modulates the balance between alternative conformations of the chromatin, histone H1 enhancing chromatin compaction, in contrast to HMGB. In *Plasmodium*, even though the gene for the H1 linker histone has not yet been annotated, along with 60% of the 5,300 predicted genes, a putative histone H1-like protein might be present and counteract the function of *Plasmodium* HMGB. Indeed, the H1 histones are evolutionarily conserved in metazoans but substantially divergent in protists (51). Some protists appeared to have only a lysine-rich basic protein, whose composition is similar to some of the histone H1-like proteins from eubacteria, animals, and plants (26).

Le Roch et al. reported differential expression of both transcripts. Pf*hmgb1* is preferentially expressed during the erythrocytic asexual stages, in contrast to Pf*hmgb2*, in which preferential expression occurs in gametocytes (35). Figure 4c shows that expression of the two corresponding proteins is closely related to the level of transcripts and is differentially expressed in mixed asexual and gametocyte stages. The Western blot of cytoplasmic and nuclear extracts prepared from asexual stages (Fig. 4b) and immunofluorescence of asexual and gametocytes stages (Fig. 5a, b, d, and e) revealed that the two factors are localized mainly in the nucleus. Furthermore, PfHMGB2 was clearly detected in the cytoplasm of gametocytes (Fig. 5d and e).

In addition to the differences in the levels of expression and localization within asexual and sexual parasites, these two factors exhibited different affinities when interacting and bending DNA (Fig. 3), PfHMGB2 being less efficient, at least when examined in vitro. All of these results argue in favor of little if any redundancy between the two proteins and in favor of a role in gametocytogenesis.

In summary, a combination of computational and molecular analyses is needed to increase our knowledge of transcriptional regulation of *Plasmodium* genes involved in crucial steps of asexual and sexual erythrocytic development. This report describes the characterization of two *Plasmodium* HMGB factors that appear to exhibit substantial similarity to architectural factors as regards their biological functions, at least when analyzed in vitro for the capacity to interact with distorted DNA and to bend DNA, even though their capacities to do so appeared to be quite different. As in eukaryotes, HMGB factors in *Plasmodium*, since they are highly conserved through evolution, are probably involved in chromatin remodeling. How-

ever, even though these proteins were observed in asexual and gametocyte stages, their levels of expression were clearly different, with PfHMGB1 likely implicated in proliferation and PfHMGB2 implicated in differentiation of *Plasmodium*. Much more work will be needed to understand the functions of these two proteins, both as nuclear factors and as cytokines. Invalidation of either the factors or the interaction between the PfHMGB and DNA via gene silencing strategies (20) and antagonists, etc., will increase our knowledge of transcriptional regulation as well as our control of the erythrocytic development of the parasite (proliferation and differentiation). It might provide exciting new therapeutic possibilities. In this regard, it is worthy of note that this type of approach, via disruption of DNA/factor interaction, is currently being evaluated for human cancer therapy with a special focus on HMG proteins (4, 40).

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