The HU Protein Is Important for Apicoplast Genome Maintenance and Inheritance in Toxoplasma gondii

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The apicoplast, a chloroplast-like organelle, is an essential cellular component of most apicomplexan parasites, including Plasmodium and Toxoplasma. The apicoplast maintains its own genome, a 35-kb DNA molecule that largely encodes proteins required for organellar transcription and translation. Interference with apicoplast genome maintenance and function is a validated target for drug therapy for malaria and toxoplasmosis. However, the many proteins required for genome maintenance and inheritance remain largely unstudied. Here we genetically characterize a nucleus-encoded homolog to the bacterial HU protein in Toxoplasma gondii. In bacteria, HU is a DNA-binding structural protein with fundamental roles in transcription, replication initiation, and DNA repair. Immunofluorescence assays reveal that in T. gondii this protein localizes to the apicoplast. We have found that the HU protein from Toxoplasma can successfully complement bacterial ΔhupA mutants, supporting a similar function. We were able to construct a genetic knockout of HU in Toxoplasma. This Δhu mutant is barely viable and exhibits significant growth retardation. Upon further analysis of the mutant phenotype, we find that this mutant has a dramatically reduced apicoplast genome copy number and, furthermore, suffers defects in the segregation of the apicoplast organelle. Our findings not only show that the HU protein is important for Toxoplasma cell biology but also demonstrate the importance of the apicoplast genome in the biogenesis of the organelle.

The phylum Apicomplexa consists of single-celled eukaryotic parasites which infect humans and many other animals. Infection with Apicomplexa causes a variety of diseases that have significant global health and economic impact. Among these parasites, perhaps the best known are Plasmodium species, the causative agents of malaria. Another prominent apicomplexan is Toxoplasma gondii. While most people do not suffer symptoms upon infection, in immunosuppressed individuals T. gondii can cause severe encephalitis, an AIDS-defining opportunistic infection. T. gondii also causes congenital disease when a woman becomes infected for the first time during pregnancy.

Most apicomplexans harbor a remnant chloroplast called the apicoplast. This plastid-like organelle, although no longer photosynthetic, still houses important biosynthetic pathways including type II fatty acid synthesis (FASH) (28), heme synthesis (39), and isoprenoid biosynthesis (34). Apicoplast pathways were shown to be essential for cell viability in T. gondii and Plasmodium (28, 34). Because this organelle is unique to these parasites and not found in the human host, apicoplast proteins and structures are considered excellent candidates as parasite-specific drug targets.

Like other chloroplasts, the apicoplast has an evolutionary history that can be traced back to cyanobacteria. Chloroplasts evolved when a eukaryotic cell engulfed a cyanobacterium, which over time underwent massive gene transfer to the host nucleus. In this process of primary endosymbiosis, the prokaryotic symbiont transformed into a subcellular organelle. Primary plastids are present in glaucophytes, plants, green algae, and red algae. Apicoplasts are likely derived from a secondary endosymbiotic event in which a second eukaryotic host engulfed a red alga and, as in the case of the cyanobacterial symbiont, over time the algal symbiont was reduced to a plastid organelle through gene transfer to the host nucleus.

Consistent with secondary endosymbiosis, the apicoplast is surrounded by four membranes and contains its own 35-kb genome that shares similarity with chloroplast genomes from red algae. Interestingly, while apicomplexan nuclear genomes display a surprising lack of synteny among more distant genera of the phylum (9), the apicoplast genome is extremely well conserved. The gene numbers and orders are almost identical in several species studied so far, including Plasmodium falciparum (54), T. gondii (J. Kissinger, personal communication), and Eimeria tenella (6). The copy number of the genome differs between species, but the T. gondii apicoplast appears to contain around 25 copies by recent estimates (27). Most of the proteins active in apicoplast metabolism, such as the enzymes in the FASII pathway, are encoded in the nucleus. The genes left behind in the apicoplast genome mostly function in apicoplast gene expression (6, 54). However, it also encodes a small number of proteins not involved in gene expression, namely, CLPC and SUFB. CLPC is a subunit of a protease known in other chloroplasts which may be involved in protein degradation or turnover, while SUFB is involved in assembly of iron-sulfur clusters.

Because loss of the apicoplast genome has been shown to be lethal to the parasite (12), proteins involved in plastid DNA replication and stability are attractive as potential drug targets. Relatively few proteins involved in these processes in Apicomplexa have been studied so far. Both A and B subunits of DNA gyrase are known to be targeted to the apicoplast (8). Their function is likely essential since the parasites are sensitive to drugs that target gyrase...
such as novobiocin and ciprofloxacin (12, 37). Recently, a gene has also been identified in Plasmodium falciparum (25, 43) and Toxoplasma gondii (33) which encodes an apicoplast-targeted multidomain protein possessing primase, helicase, and polymerase domains. The polymerase domain is related to the bacterial DNA polymerase I (Pol I) enzyme. Both apicomplexan orthologs have been shown to possess polymerase activity and have been suggested to be the replicative enzyme of the apicoplast genome, but whether it is essential for the parasite growth has not yet been demonstrated. In T. gondii there is currently no other good candidate for the replicative complex of the apicoplast DNA, but in Plasmodium there appears to be another homolog of DNA Pol I which may have an organellar localization (32). A homolog of SSB (single-stranded DNA-binding protein) which localizes to the apicoplast and binds single-stranded DNA has also been identified in Apicomplexa (36).

We were interested in identifying DNA-binding proteins in T. gondii that promote apicoplast genome stability. Since plastids are the product of an endosymbiosis of bacteria, we searched the ToxoPlasma genome database using several proteins known to associate with DNA in Escherichia coli. One of the proteins that yielded a homolog was HU. Nucleus-encoded in ToxoPlasma, HU is a histone-like protein found in bacteria as well as red algal plastids (23), and more recently in the plastid of the green alga Chlamydomonas reinhardtii (22). In bacteria, it localizes to the nucleoid (45, 53) and exists in vivo as a 20-kDa dimer. A role for HU has been demonstrated in DNA compaction and stabilization of supercoils (17), as well as in transcription (20, 21), initiation of replication (10, 42), and DNA repair (24, 30). In E. coli, ΔhuA mutants exhibit retarded growth (18) and produce a filamentation phenotype.

An HU homolog has also recently been described in Plasmodium falciparum that associates with the apicoplast and has the ability to bind and condense DNA (38). Here we investigated the role of HU in ToxoPlasma. We find that HU serves an important role in maintaining optimal levels of the plastid genome. In the absence of HU, apicoplast genome loss causes considerable defects in apicoplast biogenesis and a dramatic loss of fitness in the parasite.

MATERIALS AND METHODS

Protein purification and antibiotic production. Part of the C-terminal domain of the HU gene encoding amino acids 120 to 209 was amplified from T. gondii RH cDNA using primers I and II (see Table S1 in the supplemental material) and cloned into vector pMAL-2E (New England Biolabs) to create an N-terminal maltose binding protein fusion. Recombinant protein was produced in E. coli and purified by affinity chromatography on cross-linked amylose resin (34). Polyclonal antisera were purified from the antisera against purified HU cross-linked to activated CNBr Sepharose 4B (Sigma) as described previously (2).

E. coli complementation assays. The conserved region of the HU gene, encoding amino acids 120 to 209, was amplified using primers I and II (see Table S1 in the supplemental material) and cloned into pCR2.1-TOPO (Invitrogen). ΔhuA E. coli (a kind gift from J. Rouviere-Yaniv, Institut de Biologie Physico-Chemique, Paris, France) (4) was transformed with the resulting plasmid as well as with empty vector. Transformants were grown in LB medium with 50 μg/mL of ampicillin until they reached an optical density of 0.4 at 600 nm, at which point IPTG was added to a final concentration of 2 mM. After IPTG (isopropyl-β-d-thiogalactopyranoside) incubation, 100 μl of 106 E. coli cells/ml was allowed to settle for 15 min on coverslips coated with poly-L-lysine. Cells were fixed with 3.5% pafraormaldehyde in phosphate-buffered saline (PBS) and permeabilized with 2.5% Triton X-100 in PBS and then stained with 2 μg/mL DAPI (4′,6-diamidino-2-phenylindole) in PBS for 10 min. Cells were then imaged by fluorescence and phase-contrast microscopy on a DM IRBE inverted epifluorescence microscope (Leica) with a 100×/1.40 numerical aperture (NA) oil immersion lens as previously described (49). The area of DAPI staining was measured in individual bacterial nucleoids. Nucleoids were defined as objects by generating binary image masks for each image using a thresholding function. Student’s t test was used to compare mean nucleoid areas.

Plasmid and cosmid construction. To create constructs for HU overexpression, the HU gene was amplified by PCR from T. gondii RH cDNA using primer III and either primer IV or primer V, which encodes a stop codon (see Table S1 in the supplemental material). The resulting PCR products were then cloned into the pCR-Blunt-II-TOPO vector (Invitrogen) following the manufacturer’s instructions to create pTOPO-HU and pTOPO-HUstop. These plasmids, along with the pTubHYFP-YFP-sagCAT vector (16), were digested with BglII and AvrII, and the HU inserts were subsequently ligated into the cut yellow fluorescent protein (YFP) vector to create pTubHYFP-sagCAT and pTubHYFP-sagCAT.

To generate a construct to target and delete the TgHU gene, we modified cosmid clone TOXPJ14, which contains the TgHU locus. Briefly, primers VI and VII (see Table S1 in the supplemental material) were used to amplify a sequence containing the chloramphenicol acetyltransferase (CAT) cassette along with a gentamycin resistance marker from the plasmid pHS5G by PCR. Cosmid recombineering was employed as described previously (5) to generate the TOXPJ14-HUKO construct.

Parasite culture and genetic manipulation. For continuous culture, T. gondii tachyzoites were grown in HERT human fibroblasts in Dulbecco’s modified Eagle medium supplemented with 1% fetal bovine serum (FBS) at 37°C and 5% CO2. The parental strain of the knockout parasites, which was also used as a control in subsequent experiments, was the Δku80/TATi strain (19, 24, 44), a derivative of RH strain T. gondii. Δhu parasites were grown in the same conditions with 3% serum, and 5 ml of a 10-ml culture was passed to a new flask at about 50% lysis, which was achieved after 2 weeks in a T-25 flask.

For transfection, 107 parasites were filtered, pelleted, and resuspended in cytomix buffer (19). Thirty-five micrograms of plasmid or cosmid DNA was electroporated into parasites (1.5 kV, 25 μF, 25 Ω) using a BTX ECM 630 electronic cell manipulator. Parasites were transferred into a fresh human foreskin fibroblast culture and allowed to grow overnight before beginning drug selection. Selection of parasites for the integration of TOXPJ14-HUKO was performed using a final concentration of 20 μM chloramphenicol. Parasites were passaged twice and then cloned in 96-well plates by limiting dilution (47).

Western blotting and immunofluorescence assays. Western blotting was performed as previously described (50). HU antiserum was used at 1:200, and antitubulin antibody (a kind gift from J. Gaertig, University of Georgia) was used at 1:1,000. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies (Bio-Rad) were used at 1:20,000.

Immunofluorescence assays were performed as previously described (50) in infected human foreskin fibroblasts. The rabbit anti-HU antibody was used at 1:2,000, rabbit anti-Cnp60 was used at 1:3,000 (1), rabbit anticantrin (a kind gift from I. Cheeseman, Whitehead Institute, Boston, MA) was used at 1:500, and rabbit anti-IM1 (a kind gift from C. Beckers, University of North Carolina Chapel Hill, Chapel Hill, NC) was used at 1:1,000. Mouse anti-F1 ATPase beta subunit 5F4 (a kind gift from P. Bradley, University of California Los Angeles, Los Angeles, CA) was used at 1:1,000, mouse anti-CAT (Abcam) was used at 1:3,000, mouse antibody against the E2 subunit of pyruvate dehydrogenase (anti-PDH-E2) (a kind gift from W. Bohn, University of Göttingen, Göttingen, Germany) was used at 1:500, and mouse anti-GFP (Roche) was used at 1:200. Alexa Fluor 546-conjugated goat anti-rabbit secondary antibodies (Invitrogen) were
used at 1:200, and Alexa Fluor 488-conjugated goat anti-mouse secondary antibodies (Invitrogen) were used at 1:300.

To quantify HU-YFP staining in transient transfections with pTub-HU/YFP-sagCAT, parasites were identified by IMC1 staining and HU-YFP was visualized in fixed cells with anti-GFP staining. Apicoplasts were visualized by ferredoxin NADP$^+$ reductase-red fluorescent protein (FNRRFP) fluorescence. For each vacuole counted, the total number of parasites in the vacuole, total number of parasites containing apicoplasts, and total number of parasites expressing HU-YFP were recorded. Percentages of parasites displaying normal phenotypes were calculated, and statistical significance was assessed using Fisher’s exact test.

To quantify the number of apicoplasts or mitochondria per vacuole, 100 4-cell vacuoles were counted and numbers of apicoplasts or mitochondria per vacuole were recorded. Apicoplasts were visualized by anti-PDH-E2 staining, and mitochondria were visualized by anti-F1 ATPase staining (P. Bradley). In the comparison between Δku80/TATi and Δhu, individual parasites within vacuoles were discerned by anti-IMC1 staining of the inner membrane complex. To quantify vacuole size, 100 vacuoles containing 2 or more parasites were counted and the number of parasites per vacuole was recorded. The mean number of apicoplasts per 4-cell vacuole was also calculated and the statistical significance of differences was evaluated using Student’s t test. To quantify apicoplast-centrosome association, 100 duplicated centrosomes (stained with anticentrin) were counted and scored on whether they had localizations adjacent to part of an apicoplast (stained with anti-PDH-E2) or not. Statistical significance was assessed with Fisher’s exact test.

For drug treatments, Δku80/TATi parasites were used to infect host cells on coverslips and treated with either 10 μM ciprofloxacin or 40 ng/ml clindamycin. Coverslips were fixed at various time points (24, 48, or 72 h for apicoplast counting and 8, 16, 24, 32, 40, or 48 h for centrosome-apicoplast association scoring), and immunofluorescence assays and phenotype counting were performed as described above.

**Fluorescent in situ hybridization.** Fluorescent in situ hybridization assays were performed as described previously (11). Briefly, parasite-infected host cells grown on coverslips and treated with either 10 μM ciprofloxacin or 40 ng/ml clindamycin. Coverslips were fixed at various time points (24, 48, or 72 h for apicoplast counting and 8, 16, 24, 32, 40, or 48 h for centrosome-apicoplast association scoring), and immunofluorescence assays and phenotype counting were performed as described above.

To confirm the replacement of the native HU locus, a 389-bp probe complementary to a region upstream of the HU gene was amplified by PCR from RH genomic DNA using primers XVI and XVII (see Table S1 in the supplemental material). The PCR product was gel purified and radiolabeled with [32P]dCTP in a random priming reaction (Invitrogen). Two micrograms of genomic DNA from parental and Δhu parasites was digested with EcoRV and AvrII for the Southern hybridization.

To compare DNA levels between parental and Δhu parasites, DNA fragments were amplified by PCR from RH genomic DNA from both the nuclear UPR locus and the apicoplast genome as described previously (4). The PCR products were then radiolabeled as described above, and 2 μg of genomic DNA from parental and Δhu parasites was digested with HindIII. After hybridization, Southern blots were exposed to film for 48 h. Band intensity was quantified by densitometry using ImageJ (http://rsweb.nih.gov/ij/) from an autoradiograph, and apicoplast DNA levels were normalized to nuclear DNA levels.

**Plaque assays.** Confluent hTERT T-25 cultures were infected with 1,000 parasites from either the Δhu strain or the Δku80/TATi parental strain. After incubation for the indicated time, flask was fixed with ethanol and stained with crystal violet as previously described (47).

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Toxoplasma HU protein is targeted to the apicoplast. The N terminus of TgHU contains sequence features consistent with the presence of a bipartite signal peptide required for apicoplast localization (Fig. 1A). To establish the protein’s localization, we generated antibodies. The conserved region of the protein, amino acids 120 to 209, was amplified from *T. gondii* cDNA. This ampli-con was cloned into the pMAL-2e vector, introducing an N-terminal maltose binding protein fusion, and expressed in *E. coli*. Recombinant protein was purified and antibodies were raised in rabbits. In a Western blot of parasite lysates, the HU antiserum recognizes a band at about 16 kDa, which matches the size predicted for TgHU after removal of the transit peptide (Fig. 2A). Immunofluorescence assays using the anti-HU antibody show co-localization of the HU protein to the E2 subunit of pyruvate dehydrogenase (PDH-E2), an apicoplast marker, and to the DAPI stain of the apicoplast genome (Fig. 2B to F).

Overexpression of an HU-YFP transgene results in unequal distribution and apicoplast loss. To visualize the protein in living cells, we also attempted to tag TgHU with yellow fluorescent protein (YFP) by C-terminal translational fusion using a construct that expresses the transgene from the strong *Toxoplasma* tubulin promoter. We transfected this construct into a parasite strain expressing red fluorescent protein (RFP) fused to the signal sequence of the apicoplast protein ferredoxin NADP⁺ reductase (FNR) (46), but stable lines could not be obtained after drug selection. Fluorescence microscopy at 48 h posttransfection revealed robust transient expression of the transgene and localization of HU-YFP to the apicoplast, marked by FNR-RFP (Fig. 3A). However, the distribution appeared to be uneven—in some vacuoles expressing HU-YFP, we observed apicoplasts without any HU-YFP labeling (Fig. 3B and C). We quantified this distribution (Fig. 3G) and found that in vacuoles where HU-YFP expression was detected, 27% of the parasites were lacking HU-YFP staining. Additionally, vacuoles expressing HU-YFP had an increased incidence of apicoplast loss (as indicated by loss of FNR-RFP labeling) in one or more of the parasites. Eleven percent of transfected parasites were missing apicoplast staining, compared with less than 1% in untransfected parasites, representing a statistically significant difference (*P* = 0.0007, Fisher’s exact test). These observations suggest that the HU-YFP overexpression resulted first in unequal HU-YFP localization and then in apicoplast loss.

We noted that in transgenic parasites that display uneven distribution of HU-YFP, the DAPI staining of the apicoplast genome appeared to be similarly unequal and correlated with the HU-YFP localization: a larger, more intense spot in some parasites and a smaller, more diffuse spot in others. Therefore, we performed fluorescent in situ hybridization to specifically examine the distribution of the plastid genome in a pool of parasites transfected with HU-YFP. While in untransfected parasites, most vacuoles showed a fluorescent signal, indicating a plastid genome for each parasite, transfected parasites frequently showed vacuoles with only one or a few apicoplast nucleioids (Fig. 3E and F). Overall, this suggests that overexpression of HU-YFP causes not only improper segregation of HU-YFP but missegregation of the entire HU-genome apicoplast nucleioid. Loss of apico-
plast genome in the progeny explains why stable transgenics were not obtained.

Transfection with similar plasmids containing the HU cDNA under the control of the tubulin promoter with or without a small C-terminal myc epitope tag in place of the YFP tag also failed to generate stable lines, suggesting that overexpression of the gene may be detrimental. Selection of parasites transfected with a fusion construct utilizing a hemagglutinin (HA) tag and under the control of the T754-regulatable promoter (29), which is thought to be weaker than the tubulin promoter, resulted in a stable line characterized by mistargeting of the transgene (data not shown). Together, this suggests that the level of HU protein per cell is very important and that TgHU has a very narrow expression range in which it can carry out its function optimally.

**Loss of the Toxoplasma HU severely impairs growth.** We wanted to isolate a mutant lacking HU to study its function and the consequences of its loss. To this end, we constructed a genetic knockout in T. gondii, utilizing a cosmid recombineering strategy which results in high frequency of gene targeting (5). Briefly, a cosmid clone containing the full HU locus was selected, and the HU gene was replaced with the gene encoding the chloramphenicol acetyltransferase (CAT) drug marker. The resulting knockout cosmid was then transfected into Δku80/TATi parasites (44), which favor homologous recombination of the transfected DNA (13, 19). After selection of parasites with chloramphenicol, clones were isolated by limiting dilution. Clones were tested for gene replacement by Southern blot analysis using a probe to the 5′ noncoding region of the HU locus. Note that the 3.3-kb EcoRV/AvrII wild-type fragment is lost in the pictured clone and replaced by a 1.3-kb fragment predicted for the targeted locus (Fig. 4A and B).

We also tested for the loss of HU protein expression in the mutant. Western blotting of parental and Δhu strain lysates reveals the absence of the HU-specific band in mutant parasites (Fig. 4C). The loss of HU protein was also confirmed by immunofluorescence assay. We stained parasites with antibodies to HU and CAT (the gene for this drug resistance protein replaces the HU gene in the mutant). Parental-strain parasites showed clear HU staining and no anti-CAT fluorescence, while Δhu mutant parasites exhibited strong anti-CAT fluorescence but lacked HU staining (Fig. 4D). This demonstrated that the Δhu parasites robustly expressed the drug-selectable marker and no longer expressed the HU gene consistent with ablation of the locus.

While the ability to generate a direct HU knockout suggests that it is not an absolute requirement for parasite survival, we noticed extremely slow growth in the mutant compared to the parental strain. To examine the growth phenotype more closely, we performed a plaque assay directly comparing plaque formation of the Δhu parasites to the parental strain (Fig. 4E). In this experiment, parasites were grown in a monolayer of host cells for several days. During this time, individual parasites will invade cells in the monolayer and go through repeated cycles of invasion, cell division, and egress, creating plaques in the monolayer. By day 6 postinfection, the parental line showed detectable plaques, which proceeded to increase greatly in size through day 13. For the Δhu parasites, on the other hand, no plaques were visible at day 6, and by day 13 plaques appeared but were extremely small.

We first considered that the small plaque size we observed for the Δhu mutant may be due to a reduced rate of parasite replication resulting from impaired apicoplast function. This would be consistent with predicted functions of HU in transcription control and could for instance result from loss of transcription of critical apicoplast genome-encoded proteins. To test this, we analyzed the rate of cell division in the mutant parasites. T. gondii replicates in a binary budding process known as endodyogeny. After host cell invasion, a single parasite inhabits each parasitophorous vacuole. Successive rounds of division will result in progressive doubling of parasite numbers with an average doubling time of 6 to 8 h for the RH strain (40). We counted and recorded the numbers of parasites per vacuole at different time points postinfection for mutant and parental-strain parasites (Fig. 5), and these data were then log_{2} transformed before analysis to reflect the number of doublings. At 24 and 32 h, we see a significant difference in median parasite doublings (Table 1). These data suggest that Δhu parasites are progressing more slowly through the cell division cycle. However, this difference does not appear sufficient to account for the drastic growth retardation observed in plaque assays and cell culture.

**Loss of HU in Toxoplasma results in decreased apicoplast genome numbers.** We considered whether a significant proportion of the parasites resulting from intracellular replication might be inviable in the Δhu mutant and not able to initiate or complete another full cycle of infection. This has been observed previously as a consequence of poor inheritance of the apicoplast genome following drug treatment targeting the genome (12). As HU localizes to the apicoplast and can complement the E. coli homolog, it likely binds apicoplast DNA, as seems to be the case for its Plasmodium homolog (38). HU may be important for apicoplast DNA compaction during division and/or affect interactions of the genome with other DNA-binding proteins. To examine the consequences of a lack of HU on apicoplast genome inheritance, we examined apicoplast genome abundance by quantitative PCR on Δhu and parental-strain parasites (Fig. 6A). Using one primer pair suitable to amplify a segment of the apicoplast genome and a sec-

![FIG 2] TgHU protein localizes to the apicoplast. (A) Western blotting of RH strain parasite-derived proteins stained with antiserum raised against recombinant TgHU protein. (B to F) Immunofluorescence assay of a 4-cell vacuole in RH strain T. gondii. Staining of an antibody raised against TgHU (C) colocalized with the apicoplast marker anti-PDH-E2 (D) and the DAPI stain of the apicoplast DNA (B; note that the larger nuclear genome is also stained). Also shown are a merged image (E) and a differential interference contrast (DIC) image of the 4-cell vacuole (F).
ond primer pair specific for a single locus in the nuclear genome, we compared the copy number of apicoplast DNA to nuclear DNA. Since *Toxoplasma* tachyzoites are haploid and contain only one copy of the nuclear genome prior to S phase, this ratio should approximate the copy number of the apicoplast genome across the population. In parental-strain parasites, we measured a ratio of 21.35 ± 2.83 apicoplast genomes per nuclear genome. To demonstrate the ability of this assay to detect changes in apicoplast DNA content, we also measured the DNA content of parasites treated with 10 μM ciprofloxacin for 72 h, which has been shown in previous studies to reduce apicoplast genome copy number (12). Indeed, in ciprofloxacin-treated parasites, we observed a 51% de-

FIG 3 Overexpression of HU-YFP results in unequal plastid distribution. A construct encoding HU tagged with YFP at the C terminus was transfected into parasites expressing the apicoplast marker FNR-RFP. (A) Live imaging of RH strain *T. gondii*. HU-YFP (green) localized to the apicoplast, marked by FNR-RFP (red). A phase image of the 2-cell vacuole is shown in the rightmost panel. (B and C) HU-YFP (green, stained with anti-GFP) showed unequal distribution in divided parasites 48 h after infection. The luminal marker FNR-RFP (red) has a normal distribution in some vacuoles, with staining in every parasite (B), but vacuoles are also observed containing parasites that lack FNR-RFP staining (C). parasite outlines are stained with anti-IMC1 (blue). (D) HU-YFP is associated with the apicoplast nucleoid. HU-YFP staining (green) is not observed in every apicoplast in the vacuole (red, FNR-RFP), but where present, it localizes to apicoplasts where apicoplast DNA (white arrowheads) is also found. Nuclear and apicoplast DNA is stained with DAPI (blue). (E and F) Fluorescent in situ hybridization of transiently transfected parasites. Apicoplast DNA of fixed cells was hybridized with digoxigenin-labeled DNA probes, and immunofluorescence assays were performed using antidigoxigenin (green); counterstaining was with DAPI (blue). In control cells, a hybridization signal is observed proximal to every nucleus (E). In parasites transiently expressing HU-YFP, vacuoles with unequal hybridization signals of apicoplast DNA are frequently observed (F). Bars = 2 μm. (G) HU-YFP and FNR-RFP distribution in transfectants was quantified. By 48 h posttransfection, 10.7% of parasites lacked apicoplasts, compared to 0.5% in untransfected parasites. HU-YFP staining in transfected vacuoles was observed even less, with 26.6% of parasites in vacuoles expressing HU-YFP lacking staining. In each bar on the graph, n = 100.
crease in apicoplast DNA copy number, down to 10.46 ± 6.50 apicoplast genomes per nuclear genome. In the hu clone, we see a ratio of 2.15 ± 0.51 apicoplast genomes per nuclear genomes, which represents an 89.9% reduction (Table 2), indicating that HU loss results in a drastic decrease in apicoplast DNA levels. To independently confirm this using an assay independent of DNA amplification, we performed Southern blotting, probing for nuclear and apicoplast DNA in parental and knockout strains (Fig. 6B). Densitometry of the resulting autoradiograph shows that apicoplast DNA in the mutant is 2.4% of the wild-type level, compared to a decrease to 38.4% in ciprofloxacin-treated parasites (Fig. 6C). We conclude that HU function is critical to maintain a full complement of multiple apicoplast genomes per organelle and parasite.

Loss of HU in Toxoplasma results in an apicoplast segregation defect. We demonstrated above that overexpression of a tagged HU causes unequal apicoplast segregation, and we therefore hypothesized that the loss of HU might also result in segregation defects. To explore this possibility, we performed immunofluorescence assays on the hu knockout using an antibody to the luminal apicoplast protein Cpn60 and compared staining to parental-strain parasites. Infected host cells containing 4 daughter

![FIG 4 Deletion of the TgHU locus. (A) Diagram of the native and modified TgHU locus. Southern blotting of genomic DNA cut with AvrII and EcoRV and hybridized with a probe specific to a region upstream of the HU locus is predicted to yield a 3.3-kb band for the native locus or a 1.3-kb band for the modified locus. (B) Southern blot analysis comparing parental-strain DNA (lane P) and DNA isolated from the Δhu mutant. (C) Western blot of proteins from parental-strain and Δhu parasites. Note that the HU antiserum band is absent in Δhu lysates. Anti-alpha-tubulin (Tub) is shown underneath as a loading control. (D) Immunofluorescence assays on parental-strain (top) and Δhu (bottom) parasites, stained with anti-HU (red, middle) and anti-CAT (green, right) and shown with a DIC reference image (left). The parental strain exhibits HU staining but no CAT staining, while Δhu parasites exhibit CAT staining but lack HU staining. Bars = 2 μm. (E) Plaque assays of Δhu parasites compared to the parental strain. Parental-strain parasites yield plaques by day 6 (top left), which greatly increase in size by day 13 (top right), while no plaques can be seen in Δhu parasites on day 6 (bottom left). A few Δhu plaques (black arrow) begin to appear by day 13 (bottom right), but these are greatly reduced in size. Bar = 5 mm.

![FIG 5 Distribution of vacuole sizes in parental and Δhu parasites. Host cells were infected with parasites, and the number of parasites per vacuole was counted in 100 individual vacuoles at four different time points. One-cell vacuoles were disregarded, to avoid the inclusion of parasites that were merely attached and not yet invaded. The distribution of vacuole sizes for each strain at each time point is displayed.

| TABLE 1 Mean vacuole sizes in parental and mutant strains |
| Time (h) | Parental | Δhu mutant | P value <sup>b</sup> |
|----------|----------|-----------|---------------------|
| 8        | 1        | 1         | 1.000               |
| 16       | 2        | 2         | 0.193               |
| 24       | 3        | 2         | <0.001              |
| 32       | 4        | 3         | <0.001              |

<sup>a</sup> Parasites per vacuole were counted and transformed on a log scale to reflect the number of doublings.

<sup>b</sup> P values were calculated with the Kolmogorov-Smirnov test.
parasites were counted 16 h postinfection. In healthy parasites, we expect to see one apicoplast in every parasite, or four apicoplasts total per vacuole. In the parental strain, 96% of vacuoles had one apicoplast per cell, with only 4% of the vacuoles missing one or more apicoplasts. In contrast, in the HU knockout, 53% of vacuoles were missing at least one apicoplast, and 8% were missing all four apicoplasts (Fig. 7A to E and G). The median number of apicoplasts per vacuole was 4 for parental-strain parasites and 3 for Δhu parasites (P < 0.001, Kolmogorov-Smirnov test). This indicates that the loss of HU results in aberrant apicoplast division creating inviable parasites.

Some DNA replication proteins target to both the mitochondrion and the plastid in plants (52), and dual targeting of certain enzymes has also been observed in Toxoplasma (35). To test whether the loss of HU may impact the mitochondrion, we also counted mitochondria in 4-cell vacuoles using an antibody to the mitochondrial F1 ATPase (Fig. 7F). In Δhu parasites, 1% of vacuoles contained a parasite lacking a mitochondrion, and 6% contained punctate mitochondria instead of the typical tubular mitochondrial morphology (Fig. 7H). Since this was observed in odd-shaped cells, we suspect that this is a characteristic of dying parasites. As the majority of parasites contain normal mitochondria, it appears that loss of HU has no (primary) effect on mitochondria, supporting its plastid-specific role.

In light of the observed plastid loss, we wanted to test if segregation of the organelle might be hampered. During parasite cell division, the centrosome duplicates and facilitates the division and segregation of the nucleus and the apicoplast. During this time, the centrosome maintains a close association with the apicoplast and the nucleus (46). To investigate the plastid-centrosome associations in our mutants, we performed immunofluorescence assays staining the apicoplast marker PDH-E2 and the centrosome marker centrin and examined whether duplicated centrosomes were associated with the apicoplast. In the parental strain, duplicated centrosomes were closely associated with apicoplasts, but in the Δhu mutant we found increased numbers of parasites whose centrosomes did not seem to exhibit any apicoplast association (see Fig. S1A in the supplemental material). We quantified these associations, and in the Δhu mutant 33% of the duplicated centrosomes lacked apicoplast associations, compared with only 18% in the parental strain (see Fig. S1B). This difference was statistically significant (P = 0.0079, Fisher’s exact test) and indicates that loss of HU decreases the efficiency of centrosome association for the apicoplast, which contributes to unequal apicoplast segregation. Note that this observation does not rule out additional defects in apicoplast biogenesis.

While HU loss appears to reduce the efficiency of apicoplast segregation and cause plastid loss, the mechanism by which this occurs is unclear. One possibility is that the reduction in apicoplast genome copy number leads to reduced expression of apicoplast-encoded proteins important for biogenesis and division. Alternatively, the apicoplast DNA may be physically required for centrosome association, e.g., as a special marker of the site of attachment. To attempt to distinguish between these two scenarios, we compared parasites in which we inhibited either apicoplast or mitochondrial enzymes has also been observed in Toxoplasma (35). To test whether the loss of HU may impact the mitochondrion, we also counted mitochondria in 4-cell vacuoles using an antibody to the mitochondrial F1 ATPase (Fig. 7F). In Δhu parasites, 1% of vacuoles contained a parasite lacking a mitochondrion, and 6% contained punctate mitochondria instead of the typical tubular mitochondrial morphology (Fig. 7H). Since this was observed in odd-shaped cells, we suspect that this is a characteristic of dying parasites. As the majority of parasites contain normal mitochondria, it appears that loss of HU has no (primary) effect on mitochondria, supporting its plastid-specific role.

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![FIG 6](https://example.com/fig6.png)

**FIG 6** Δhu parasites display reduced apicoplast genome copy numbers. (A) Quantitative PCR was performed on mutant and parental-strain parasites using separate primer pairs annealing to the apicoplast or nuclear genome. Results are presented as the ratio of apicoplast DNA copies to nuclear DNA copies. In addition to parental-strain parasites and Δhu parasites, parental-strain parasites treated for 72 h with 10 μM ciprofloxacin are also shown for comparison. Apicoplast DNA levels of the Δhu parasites are statistically significant compared to untreated parental-strain parasites (**, P < 0.005, Student’s t test). (B) Southern blot showing parasite genomic DNA products for nuclear and apicoplast DNA as independent confirmation of the quantitative PCR phenotype. (C) Densitometry on Southern blot after normalization of apicoplast DNA levels to nuclear DNA levels. Values are shown as percentages of untreated parental-strain parasites (**, P < 0.005, Student’s t test) and indicate that the loss of HU decreases the efficiency of centrosome association for the apicoplast, which contributes to unequal apicoplast segregation. Note that this observation does not rule out additional defects in apicoplast biogenesis.

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| Strain                  | Mean ratio | Decrease (%) | P value | Southern normalization |
|------------------------|------------|--------------|---------|-----------------------|
| Parental, untreated    | 21.35 ± 2.83 | 100.0       | 38.4    | 2.4                   |
| Parental, treated      | 10.46 ± 6.50 | 51.0       | 0.145   | 38.4                   |
| Δhu mutant             | 2.15 ± 0.51  | 89.9        | 0.003   | 2.4                   |

*Mean ratio of copy number of apicoplast DNA to copy number of nuclear DNA from quantitative PCR analysis.

**Relative to untreated parental DNA samples.

*P values were calculated using Student’s t test, comparing mean ratios to untreated parental samples.

*Apicoplast band intensities on Southern blot were normalized to nuclear band intensities and expressed as a percentage of untreated parental levels.
The histone-like HU protein serves to compact the genomes of bacteria and algal chloroplasts, mainly of those in the red lineage. In bacteria, HU has also been shown to maintain negative supercoiling of genomic DNA, which can influence a variety of processes including initiation of replication, transcription, and DNA repair (10, 24, 30, 42). We demonstrate here that Toxoplasma gondii encodes an HU homolog that is targeted to the apicoplast, and we study its molecular function analyzing bacterial and parasite mutants.

The primary function of the E. coli HU protein is to condense DNA and stabilize negative supercoils in the circular chromosome (41), thereby regulating DNA topology. Our experiments show that the Toxoplasma HU is able to complement the loss of HU in bacteria. This suggests that TgHU serves a similar condensation function. This is consistent with biochemical studies which demonstrated that recombinant HU from Plasmodium falciparum (PF41) is capable of condensing plasmid DNA in vitro (38). Like its Toxoplasma homolog, the Plasmodium HU protein was shown to localize to the apicoplast. Our in vivo measurements further establish a dramatically reduced copy number of the apicoplast genome in the Δhu mutant. This argues for defects in DNA replication as a result of lacking HU activity. Taken together, this indicates that proper DNA compaction is necessary for efficient replication and inheritance of the genome. Recent studies on the HU homolog HLP in Chlamydomonas reinhardtii show that RNA interference (RNAi) knockdown of HLP similarly results in reduced chloroplast nucleoid content (22) in the green chloroplast lineage. This is consistent with our results and suggestive of a common role for HU in algal plastids across the evolutionary spectrum (note that HU is not found in chloroplasts of plants).

While the low apicoplast genome copy number we observed in the Toxoplasma Δhu mutant indicates defects in DNA replication, it does not rule out additional defects in DNA segregation. Such defects have been observed in bacterial HU and DNA gyrase mutants, leading to a model in which genome segregation requires an optimal level of DNA compaction achieved through the collective and balanced activity of these proteins (51). How exactly HU impacts segregation is not fully understood, but the level of compaction of the chromosome could impact the binding of other proteins involved in the segregation process. While DNA replication requires direct access to stretches of single-stranded DNA, many other DNA-binding proteins bind to the major or minor grooves of the DNA double helix, and these interactions may be affected by altered condensation, writhe, or bending of the DNA.
titioning system of the plasmid P1, for example, the efficiency of partitioning is increased when DNA is bent by IHF (14), a paralog of HU also involved in bacterial DNA condensation.

How genome segregation and overall division are coordinated during the division of bacteria and bacterially derived organelles like mitochondria and plastids remains an active area of research and debate. In apicomplexans, the apicoplast occupies a subcellular location apical to the nucleus and close to the centrosome. Upon duplication of the centrosome, the apicoplast shows tight physical association with both daughter centrosumes and elongates as the centrosumes move apart (46). The elongated organelle is then constricted in a cytokinetic ring unique to the apicomplexan budding process. MORNI1 is a key protein of this ring and is required for optimal apicoplast division (26). The final step of apicoplast fission depends on the dynamin-like protein DrpA (49). Overall, therefore, apicoplast division is ruled by eukaryotic mechanisms, and organelle fission and segregation are accomplished and positioned by factors found outside of the apicoplast in the cytoplasm, most importantly the centrosome (46).

It is not known what role, if any, the apicoplast DNA plays in this process. In bacteria, the chromosome seems to play a direct role in cell division. The genome exclusion hypothesis postulates that proteins such as FtsZ that define the cellular point of fission preferentially localize to areas of the cell devoid of DNA (3, 7). Thus, the position of the two segregated nucleoids at opposite poles of the cell may contribute to initiation of the division furrow at a midcell location. FtsZ and several associated factors are found in essentially all primary and secondary chloroplasts studied so far (31). While there are some eukaryotic elements of plastid division, it appears that the event is controlled and initiated from the inside out, with the bacterial machinery in the lead to define the point of fission. In contrast, FtsZ and other bacterial division proteins are markedly absent in Apicomplexa (48). In parasites overexpressing HU-YFP, we observed individuals that possessed an apicoplast but lacked detectable nucleoid staining. This suggests that the apicoplast DNA is not a sine qua non requirement for organelle division and that the nucleoid may not be as important in this process as its counterpart in bacterial division (15).

However, we note that extended periods of loss of HU results not only in the loss of the apicoplast genome but also in the loss of the entire organelle, possibly due to unequal segregation. Similar to our knockout mutants, sustained overexpression of HU-YFP also resulted in apicoplast loss. This strongly suggests that while not acting in the immediate spatial control of organelle division, factors on the inside of the apicoplast are ultimately required for its biogenesis and inheritance. This is consistent with the phenotype of several mutants in apicoplast protein import and metabolism (1, 28, 50). Loss of the organelle is a secondary long-term consequence in most of these mutants. We therefore consider loss of the apicoplast genome a primary phenotype and loss of the entire organelle a secondary phenotype of the loss of HU.

Overall, our experiments show that the level of HU in the apicoplast is extremely important and that the parasite tolerates changes only within a very narrow range. Based on our findings and additional studies on bacterial HU, binding of HU to DNA and the resulting compaction likely has a major effect on the ability of other DNA-associated proteins to interact with the genome. Ultimately, this means that HU binding impacts a variety of vital processes like DNA replication, transcription, and DNA repair. Additional studies are needed to understand whether the role of HU has a purely structural function in apicoplast DNA topology or whether, not unlike eukaryotic histone proteins, HU also has important regulatory roles in the biology of the apicoplast genome.

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