Efficient Gene Replacements in *Toxoplasma gondii* Strains Deficient for Nonhomologous End Joining

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A high frequency of nonhomologous recombination has hampered gene targeting approaches in the model apicomplexan parasite *Toxoplasma gondii*. To address whether the nonhomologous end-joining (NHEJ) DNA repair pathway could be disrupted in this obligate intracellular parasite, putative KU proteins were identified and a predicted KU80 gene was deleted. The efficiency of gene targeting via double-crossover homologous recombination at several genetic loci was found to be greater than 97% of the total transformants in KU80 knockouts. Gene replacement efficiency was markedly increased (300- to 400-fold) in KU80 knockouts compared to wild-type strains. Target DNA flanks of only ~500 bp were found to be sufficient for efficient gene replacements in KU80 knockouts. KU80 knockouts stably retained a normal growth rate in vitro and the high virulence phenotype of type 1 strains but exhibited an increased sensitivity to double-strand DNA breaks induced by treatment with phleomycin or γ-irradiation. Collectively, these results revealed that a significant KU-dependent NHEJ DNA repair pathway is present in *Toxoplasma gondii*. Integration essentially occurs only at the homologous targeted sites in the KU80 knockout background, making this genetic background an efficient host for gene targeting to speed postgenome functional analysis and genetic dissection of parasite biology.

*Toxoplasma gondii* is a widespread obligate intracellular protozoan pathogen of virtually all warm-blooded animals and commonly infects humans worldwide. Due to a significant menu of established experimental approaches, *T. gondii* has become a model for the study of closely related disease-causing parasites (*Plasmodium*, *Theileria*, and *Cryptosporidium* species) belonging to the phylum *Apicomplexa* (32) and a model for the study of intracellular pathogens (33). Unfortunately, a high frequency of nonhomologous recombination arising from a previously undetermined double-strand break (DSB) DNA repair pathway(s) has hampered gene targeting approaches in this model.

DSB repair in most eukaryotes occurs primarily via two different recombination pathways (27). The homologous recombination pathways repair a DSB using mechanisms that recognize highly homologous DNA sequences, while the nonhomologous end-joining (NHEJ) pathway does not rely on DNA sequence homology. Instead, NHEJ involves direct ligation of the ends of broken DNA strands. KU70 and KU80 proteins form a heterodimer that tightly binds the DNA ends at the DSB, an early and essential step of NHEJ (49, 50). In addition to KU70 and KU80 proteins, NHEJ is mediated by the DNA ligase IV-Xrcc4 complex and the DNA-dependent protein kinase catalytic subunit or other functionally equivalent protein complexes (49).

Many eukaryotes preferentially use the NHEJ pathway to repair a DSB, and exogenous targeting DNA can be integrated anywhere into the genome independent of DNA sequence homology (27). The NHEJ pathway also appears to be preferentially used by *Toxoplasma gondii* based on the high rates of nonhomologous recombination and low gene targeting frequencies observed experimentally (7, 15, 26, 45). It is interesting that in contrast to the prevalence of the NHEJ pathway in eukaryotes, no functional NHEJ pathway has previously been reported in any protozoan parasite. Kinetoplastids, including *Trypanosoma* and *Leishmania* species, naturally exhibit extremely efficient homologous recombination of exogenous DNA (5, 12). While kinetoplastids possess the KU70 and KU80 components of the NHEJ pathway, recent evidence suggests that the NHEJ pathway is functionally absent (1, 24), although KU proteins do participate in telomere maintenance in *Trypanosoma brucei* (4, 30). *Plasmodium* species lack identifiable genes encoding key components of the NHEJ pathway and also exhibit a high frequency of homologous recombination (21, 51).

While most fungal organisms exhibit a low or modest gene targeting frequency, homologous recombination dominates at essentially a 100% frequency in *Saccharomyces cerevisiae* (22), making this budding yeast a significant model organism. Recent studies have reported that much higher frequencies of gene targeting are observed in mutants of *Aspergillus niger* (39), *Cryptococcus neoformans* (25), *Kluyveromyces lactis* (35), *Neurospora crassa* (29, 41), and other fungal organisms that have been engineered to be deficient in NHEJ. NHEJ-deficient fungal strains have greatly accelerated the development of genome-wide knockouts (3, 11).
It was previously unknown whether a high frequency of nonhomologous recombination in *T. gondii* was due to KU-dependent NHEJ or arose from other potential mechanisms of DSB repair. In this report KU80 is found to be an essential component of a functional NHEJ pathway. KU80 knockouts now allow the efficient targeting of gene replacements, gene knockouts, and gene “knock-ins” in *Toxoplasma gondii*.

**MATERIALS AND METHODS**

**Primers.** All oligonucleotide primers used in this study are listed in Table S1 in the supplemental material.

**Plasmid constructs.** Plasmids were based on pCR4-TOPO (Invitrogen), except for plasmid pC4HX1-1, which was based on a pET41 vector (20).

Plasmid pmin31-X2-4(c) was constructed from plasmid pminCAT/HX (6, 7), in which the CAT gene was replaced with the coding region for cytosine deaminase (CD) (14) (see Table S1 in the supplemental material).

Plasmid pKU80HXFCD was constructed by fusing, in order, a 52-kb 5′ KU80 target DNA flank from plasmid pAN142, the HXGPRT cassette (forward orientation) obtained from plasmid pmin31-X2-4(c), a 4.8-kb 3′ KU80 target flank from plasmid pPN111, and a downstream CD-negative selectable marker (see Table S1 in the supplemental material). The 5′ and 3′ KU80 target flanks in plasmid pKU80HXFCD surround a ~4-kb deletion of the KU80 gene (2 kb 5′ untranslated region [UTR], exon 1, intron 1, and exon 2).

Plasmid pKU80HXFCD was based on plasmid pKU80HXFCD, in which the HXGPRT marker and part of the 5′ and 3′ target flanks were deleted by digestion with BamHI. Plasmid pKU80HXFCD contains 3.3-kb (5′) and 2.4-kb (3′) KU80 target flanks that surround an ~8-kb deletion of the KU80 gene (5′ UTR and all coding exons).

Plasmid pKU80HXFCD was based on plasmid pKU80HXFCD, in which NheI was used to further delete the KU80 target flanks to 1.4 kb (5′) and 0.9 kb (3′) and join (forward orientation) the DHFRTKTS marker obtained from pDHFRTKTS (13).

Plasmid pKU80HXFCD was constructed by PCR to join a 1.1-kb UPRT 5′ target and a 0.67-kb 3′ UPRT target flank that surrounded a deletion of the UPRT coding region. The 5′ and 3′ UPRT target flanks in plasmid pKU80HXFCD surround a ~4.4-kb deletion of the UPRT gene that deletes 0.9 kb of 5′ UTR and the first six exons of UPRT.

Plasmid pKU80HXFCD was constructed from plasmid pKU80HXFCD by inserting the DHFRTKTS marker in the forward orientation between the 5′ and 3′ UPRT target flanks. Plasmid pKU80HXFCD was isolated, except the 3′ UPRT target flank was 0.54 kb.

Plasmid pC4HX1-1 was constructed from plasmid pC4 (20), which contains the coding Cassi cDNA under the control of authentic Cassi 5′ and 3′ UTR, by adding the 1.9-kb DHFRTKTS marker in the forward orientation downstream of the Cassi 3′ UTR.

Plasmids of the pHXH-series were constructed by PCR (see Table S1 in the supplemental material), and products were cloned into pCR4-TOPO. Plasmids pHXH-0, pHXH-50, pHXH-120, pHXH-230, pHXH-450, pHXH-620, and pHXH-910 contained the 1.5-kb DHFRTKTS Sall fragment (containing the last 89 codons of DHFRTKTS plus 3′ UTR) surrounded by 0, ~50-bp flanks, ~120-bp flanks, ~230-bp flanks, ~450-bp flanks, ~620-bp flanks, and ~910-bp target DNA flanks, respectively. All pHXH series plasmids were orientated in the forward orientation relative to a unique Pmel site in pCR4-TOPO.

**Strains and culture conditions.** The parental strains of *Toxoplasma gondii* used in this study were RH (46) and RHΔexogpt (6). A list of strains used in this study is shown in Table 1. Parasites were maintained by serial passage in diploid human foreskin fibroblasts at 36°C (15).

**Genomic DNA isolation and PCR.** Genomic DNA purifications used the DNA Blood minikit (Qiagen). PCR products were amplified using a mixture (1:1) of Taq DNA polymerase and Expand long template PCR reagent (Roche). Real-time PCR used various concentrations of parasite DNA (1, 10, 100, 1,000, and 10,000 pg) amplified (in triplicate) with either the *T. gondii* B1 gene primer pairs B1F and B1R at 10 μM of each primer per reaction mixture (34) or using a primer pair specific to KU80 (primers Ku80RTP and Ku80RTR). Amplification was performed by real-time PCR (Cepheid Smart Cycler) using one thermophilized SmartMix bead (SmartMix HM; Cepheid) per mixture. Each reaction mixture contained 1:20,000 SYBR Green I (Cambi BireScience). Parasite genome equivalents were determined by extrapolation from a standard curve using RHΔexogpt DNA.

**Transformation of *Toxoplasma gondii* and knockout verification strategy.** Electroporations (using a model BTX600 electroporator) were performed in 0.4 ml corning electroporation buffer containing 1.133× 107 freshly isolated tachyzoites and 15 μg of DNA (10, 31). In gene replacement experiments the targeting plasmid was linearized 5′ of the targeted DNA flank. Following selection of parasite clones, the genotype of clones was determined by PCR to measure (i) loss of the deleted coding region of the targeted gene, (ii) correct targeted 5′ integration, (iii) correct targeted 3′ integration, and (iv) the presence of a targeted DNA flank. DNA sequence analysis and real-time PCR were also used for verification of knockouts.

**KU80 knockout.** Strain RHΔku80::HXGPRT was constructed from RHΔexogpt by integration of the HXGPRT marker using plasmid pKU80HXFCD. Following transfection of tachyzoites with plasmid pKU80HXFCD, parasites were selected in mycophenolic acid (MPA; 25 μg/ml) and xanthine (50 μg/ml) (6). Negative selection experiments used fluocytosine (5FC; 50 μM) (14). Parasite clones were isolated by limiting dilution (15). Verification of disruption of the KU80 locus with the HXGPRT marker was performed by PCR with five sets of primers: PCR 1 used D801F and D801R, PCR 2 used EX801F and EX801R, PCR 3 used HXDF1 and HXDR2, PCR 4 used 80RTF and 80RTR, and PCR 5 used CDXF1 and CDXR2. The spontaneous reversion frequency of strain RHΔku80::HXGPRT was measured in 6- thioxanthine (6TX; 200 μg/ml) (43). Strain RHΔku80::HXGPRT was constructed by targeted deletion of the HXGPRT marker using plasmid pKU80HXFCD and 6TX selection. Verification of removal of the HXGPRT marker from the KU80 locus was performed by PCR with four sets of primers: PCR 2 used EX801F and EX801R, PCR 3 used HXDF1 and HXDR2, PCR 6 used EX802F and EX802R, and PCR 7 used 80f15 and 80RC3.

**Determination of gene replacement frequencies and statistical analysis.** PFU assays were used to determine absolute numbers of PFU that developed under different selection conditions, and then a formula was applied to calculate the gene replacement frequency (GRF) at the targeted locus. Four replicates PFU assays were performed for each selection point and each selection condition. A Student t test analysis was used to calculate the standard error of the mean.

**Gene replacement at the KU80 locus.** Strain RHΔexogptΔku80::DHFRTKTS was constructed from strain RHΔexogpt::DHFRTKTS by integration of the DHFRTKTS marker using plasmid pKU80HXFCD. Following transfection of tachyzoites with plasmid pKU80HXFCD, parasites were selected in pyrimethamine (PR; 1 μM). Verification of replacement of the DHFRTKTS marker in the KU80 mutant was performed with PCR with five sets of primers: PCR 1 used HXDF1 and HXDR2, PCR 2 used EX801F and EX801R, PCR 3 used EX803F and EX803R, PCR 4 used TKXF1 and TKXR2, PCR 5 used CDXF1 and CDXR2, and PCR 6 used 80FCX3 and 80RC3. PFU assays were performed at 15 days after transfection to determine the GRF based on the

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**TABLE 1. Strains used in this study**

| Strain | Parent | Genotype | Source or reference |
|--------|--------|----------|---------------------|
| RH    | RH(ERP) | Wild type | 44, 46 |
| RHΔexogpt | RH | Δhxgpt | 6 |
| RHΔku80::HXGPRT | RHΔexogpt | Δku80::HXGPRT | This study |
| RHΔexogptΔku80::DHFRTKTS | RHΔexogptΔku80::DHFRTKTS | This study |
| RHΔku80::HXGPRT | RHΔhxgpt | Δku80 | This study |
| RHΔku80::HXGPRT | RHΔhxgpt | Δku80 | This study |
| RHΔku80::HXGPRT | RHΔhxgpt | Δku80 | This study |
| RHΔnxgptΔpsI::CPSII-DNAHXGPRT | RHΔku80::HXGPRT | Δku80ΔpsI::CPSII-DNAHXGPRT | This study |
FIG. 1. Construction of T. gondii strains in which KU80 is disrupted. (A) Strategy for disrupting the KU80 gene via integration of the HXGPRT marker into strain RHΔhxgprt. Targeting plasmid pΔKUHXFCD targets a ~4-kb deletion of the KU80 gene (see Materials and Methods). Parasites were selected by positive selection in MPA plus xanthine or by negative selection against the downstream cytosine deaminase (CD) marker in MPA plus xanthine plus flucytosine.
fraction of parasites with dual resistance to PYR (1 μM) and 6TX compared to parasites with resistance only to PYR.

Gene replacement at the **UPRT** locus. Strain RHΔku80::Δuprt was constructed from RHΔku80::Δuprt by targeted integration of the HXGPRT marker using plasmid pUP-ΔHX5 or pUP-ΔHXB. Following transfection with plasmid pUP-ΔHX5 or plasmid pUP-ΔHXB, parasites were selected in MPA and then cloned. Verification of disruption of the **UPRT** locus was performed by PCR with four sets of primers: PCR 1 used DUPRF1 and DUPRR1, PCR 2 used UPFN1 and UPNXR1, PCR 3 used 3’DHFRCFX and 3’CPX MUPR15, and PCR 4 used 5’UPNCXFX and 5’DFHCXFR. PFU assays were performed at various times after transfection to determine the GRF based on the fraction of parasites that had dual resistance to MPA and 5-fluorodeoxyuridine (FUDR; 5 μM) compared to the fraction of parasites that were resistant only to MPA. Strain RHΔku80::Δuprt was constructed by targeted deletion of the HXGPRT marker using plasmid pUPNC. Following transfection with plasmid pUPNC, parasites were continuously selected in 6TX. Validation PCRs used primer pairs 3’DHFRCFX and 3’CPX MUPR1 and CLUPF1 with 3’CPX-MUPR1.

**Gene replacement at the CPSII locus.** Strain RHΔku80::Δpspi was constructed from RHΔku80::Δpspi by integration of the HXGPRT marker using plasmid pC4HX1-1. Verification of deletion of the endogenous CPSII locus and replacement with a functional CPSII DNA was performed by PCR with four sets of primers: PCR 1 used CPSDF1 and CPSDR1, PCR 2 used CPSSEX1 and CPSSEXR1, PCR 3 used CPSCEF1 and CPSCECR1, and PCR 4 used 3’DHFRCFX and CPSXCR2.

**Chromosomal repair of Δhxgprt.** Strain RHΔku80 was constructed from RHΔku80::Δhxgprt by repair of the Δhxgprt chromosomal locus using pHX plasmids with various lengths of homologous DNA that flanked a 1.5-kb SaI fragment which had been deleted in the Δhxgprt background (6). Verification of chromosomal repair of HXGPRT was performed by PCR with primers HFXI200 and HXDR2. The frequency of chromosomal repair events at the HXGPRT locus was determined in PFU assays in a single 150-cm² flask of human foreskin HXGPRT and HXDR2. The frequency of chromosomal repair events at the HXGPRT locus was then calculated based on comparison to results from the pHXH-620 plasmid, which carried the target DNA flanks. The fraction of tachyzoites surviving transfection was measured in each transfection experiment in a PFU assay. Plasmid pHXH-620 was used to determine the percent maximal chromosomal recombination as a function of DNA concentration or conformation.

**Parasite growth rate.** Tachyzoite growth rate was determined by scoring 50 randomly selected vacuoles as previously described (15).

**Sensitivity to chemical mutagens and phleomycin.** Sensitivity to N-nitroso-N-ethyleurea (ENU; Sigma) was determined by treatment of intracellular parasites using previously described methods (44). Etoposide (Sigma) sensitivity was determined using plasmid pC4HX1-1. Verification of deletion of the endogenous CPSII locus and replacement with a functional CPSII DNA was performed by PCR with four sets of primers: PCR 1 used CPSDF1 and CPSDR1, PCR 2 used CPSSEX1 and CPSSEXR1, PCR 3 used CPSCEF1 and CPSCECR1, and PCR 4 used 3’DHFRCFX and CPSXCR2.

**RESULTS**

**Generation of T. gondii strains RHΔku80::HXGPRT and RHΔku80::Δhxgprt.** To look for potential components of the NHEJ pathway in T. gondii the ToxoDB genome database (http://www.toxodb.org/toxo; release 3.0) was scanned for potential KU70 and KU80 proteins. BLASTp analysis of the KU70 (mus51) and KU80 (mus52) proteins of *Neurospora crassa* identified T. gondii homologs encoded by genes at loci 50.m03211 and 583.m05492, respectively (alignments are shown in Fig. S1 and Fig. S2 in the supplemental material).

Circular or linearized plasmid pΔKU80HXFCD was transfected into strain RHΔhxgprt and parasites were selected in MPA (Fig. 1A). Transfection with circular plasmids produced no KU80 knockouts (0/36 [data not shown]), while transfections with linearized plasmids produced 11/36 clones that showed a correct PCR 2 product but no detectable PCR 1 product that corresponded to the deleted region and identified KU80-disrupted clones (Fig. 1B). Real-time PCR analysis (PCR 4) of KU80 gene copy number indicated that ~82% of the KU80-disrupted clones contained a single copy of the KU80 3’ target DNA (Fig. 1A and B). In KU80-disrupted clones, PCR 3 was positive (presence of HXGPRT marker) and PCR 5 was negative (absence of the CD marker) (data not shown). These results suggested that the HXGPRT marker was inserted into a correctly disrupted KU80 locus (Fig. 1B).

A downstream CD marker on plasmid pΔKU80HXFCD was tested in a negative selection strategy using 5FC to enrich for the desired KU80 knockouts (Fig. 1A). Transfected parasites were initially selected in MPA for 10 days, then were cloned in MPA plus 5FC to counterselect against the downstream CD gene (Fig. 1A). We observed that six of six analyzed clones were KU80 knockouts (data not shown).

To recover the HXGPRT marker from the KU80 locus, clones of strain RHΔku80::HXGPRT were transfected with plasmid pΔKU80B (Fig. 1C) and nine 6TX-resistant clones were obtained from each transfection. Each 6TX-resistant clone had the genotype Δku80Δhxgprt (Fig. 1D), based on the presence of correct product from PCR 6 and the absence of any product from PCR 2 (Fig. 1C and D). PCR 3 also revealed the expected loss of the HXGPRT marker, and PCR 7 produced a correct product (~2.9 kb) showing targeted integration of the 3’ target DNA flank (data not shown).
Growth, virulence, and sensitivity of *T. gondii* strains RHΔhxgprt, RHΔku80::HXGPRT, and RHΔku80Δhxgprt to DNA damaging agents. Disruption of KU70 or KU80 typically induces an increased sensitivity to DNA damaging agents, particularly to agents that cause double-strand DNA breaks (25, 35, 39). Tachyzoite growth rate, sensitivity to ENU, and sensitivity to etoposide were unchanged between strains RHΔku80::HXGPRT and RHΔku80Δhxgprt compared to RHΔhxgprt or RH (data not shown). The higher virulence of type I strains was also retained in the KU80 knockout mutants (Fig. 2A). In contrast, phleomycin treatments of 5 μg/ml or 50 μg/ml reduced parental RHΔhxgprt viability to 56% (±16%) and 21% (±9%), reduced strain RHΔku80::HXGPRT viability to 19% (±8%) and 19% (±0.05%), and reduced strain RHΔku80Δhxgprt viability to 14% (±3%) and 0.23% (±0.02%), respectively (Fig. 2B). KU80 knockouts were 2.9- to 4.0-fold more sensitive to 5 μg/ml and 9- to 11-fold more sensitive to 50 μg/ml phleomycin treatment than the parental strains. Sensitivity to γ-irradiation was also significantly increased in KU80 knockouts. At a dose of 35 Gy, the percent parasite viability of strain RHΔhxgprt was 5.4% (±1.1%), viability of strain RHΔku80::HXGPRT was 0.013% (±0.005%), and viability of strain RHΔku80Δhxgprt was 0.010% (±0.003%) (Fig. 2C). KU80 knockouts were between 415- and 540-fold more sensitive to γ-irradiation (35-Gy dose) than the parental strains.

**GRF at the KU80 locus.** A simple PFU assay strategy was devised to measure the percentage of gene replacement events at the disrupted KU80 locus. This strategy (see Materials and Methods) targeted the replacement of the HXGPRT gene (inserted at the KU80 locus in strain RHΔku80::HXGPRT) with the trinfectional *DHRRTKTS* gene. Using target DNA flanks of only 1.3 and 0.9 kb carried on targeting plasmid pΔKU80TKFCD, the efficiency of gene replacement at the KU80 locus was 97.1% (±2.3%) (data not shown).

**GRF at the uracil phosphoribosyltransferase locus.** To measure gene replacement efficiency in KU80 knockout compared to parental strains, we targeted the disruption of the *UPRT* locus. Loss of uracil phosphoribosyltransferase (UPRT) function in any genetic background results in resistance to FUDR (7, 9, 42). A fixed 5′ target DNA flank of 1.3 kb and either a 0.54-kb (plasmid pΔUPT-HXS [not shown]) or a 0.67-kb (plasmid pΔUPT-HXB) 3′ target DNA flank were used in the UPRT disruption assay (Fig. 3A). The frequency of gene replacement (UPRT knockout) was determined at different time points after transfection by plating equal numbers of parasites in either MPA or MPA plus FUDR selection (Fig. 3B). The frequency of gene replacement at the UPRT locus in strain RHΔku80Δhxgprt was 99.8% (±0.6%) when assayed at 20 days posttransfection (Table 2). Similar results were observed using plasmid pΔUPT-HXS (data not shown). The comparative gene replacement percent efficiency in the parental strain RHΔhxgprt was 0.30% (±0.04%). The relative efficiency of gene replacement was enhanced by 300- to 400-fold at the UPRT locus in strain RHΔku80Δhxgprt compared to the efficiency measured in the parental strain RHΔhxgprt.

The nonreverting genotype Δku80 Δuprt::HXGPRT was confirmed in several MPA-resistant clones (Fig. 3C). A cleanup vector, pΔUPNC, containing 5′ and 3′ *UPRT* target DNA flanks but no *HXGPRT* marker, was then used to target the removal of the integrated *HXGPRT* marker from the *UPRT* locus in a clone of strain RHΔku80Δuprt::HXGPRT to generate strain RHΔku80ΔuprtΔhxgprt (data not shown).

**Gene replacement at the carbamoyl phosphate synthetase II (CPSII) locus.** Our previous work revealed a very low (~0.2%) frequency of gene targeting at the essential *CPSII* locus (15).
Gene replacement efficiency at the CPSII locus was examined in strain RHΔku80Δhxgprt using a direct replacement knock-in strategy (Fig. 4A). Target flanks of 1.5 kb 5′ and 0.8 kb 3′ that surrounded the CPSII cDNA minigene and an HXGPRT marker (plasmid pC4HX1-1) were used to delete the endogenous CPSII gene and replace it with a functional CPSII cDNA (CPSII^{DNA}) (20). Each MPA-resistant clone failed to produce any PCR 1 product specific to intron 27 of the endogenous CPSII locus and using primers positioned within exons 21 and exon 23 (PCR 2) produced a PCR product that corresponded to MPA and FUDR (5 μM) compared to the fraction of parasites that were resistant to MPA (Table 2). (C) Genotype verification of clones selected for MPA resistance after transfection with the pΔUPT-HXB plasmid. For parental strain RHΔhxgprt, PCR 1 was positive (304-bp product; lane b), PCR 2 was positive (460-bp product; lane C), and PCR 3 was negative (840-bp product; lane a). For MPA-resistant clones isolated after transfection with plasmid pΔUPT-HXB, PCR 1 was negative, PCR 2 was positive, and PCR 3 was positive. Control lane (c) contained no template and the PCR 1 and PCR 2 primers. Twelve of 12 MPA-resistant clones revealed targeted gene replacement at the UPRT locus.

Parameters affecting the efficiency of gene targeting in KU80 knockouts. In fungal strains deficient for NHEJ, the overall gene targeting efficiency is increased due to a marked reduction in the frequency of nonhomologous recombination, rather than to any significant increase in the efficiency of homologous recombination (25, 35, 39, 41). To verify that loss of nonhomologous recombination is the explanation behind increased gene targeting efficiency in KU80 knockouts versus the alternative mechanism, that homologous recombination efficiency is increased, we devised a novel strategy to specifically and quantitatively measure homologous recombination (only double-crossover events). To verify that loss of nonhomologous recombination event (even if they occur), because each targeting plasmid carries only a fragment of the HXGPRT gene that cannot confer MPA resistance in the Δhxgprt background. Double-crossover homologous recombination mediated by targeting DNA flank lengths of 0, 50, 120, 230, 450, 620, and 910 bp was examined. At each target DNA flank length (except 230 bp) the efficiency of gene replacement via double-crossover homologous recombination at the HXGPRT locus was similar (within twofold on a per parasite basis [data not shown]) between strain RHΔku80Δhxgprt and the parental strain, RHΔhxgprt (Fig. 5B). These results, along with data shown in Fig. 2, 3, and 4 and Table 2, reveal that the increased gene targeting efficiency in KU80 knockouts arises from the loss of a major nonhomologous recombination pathway rather than from any significant increase in the efficiency of homologous recombination.

The minimal target DNA flank length for gene replacement at the HXGPRT locus was determined (Fig. 5B). No gene replacement events were detected in strain RHΔhxgprt when we used 230-bp target DNA flanks. While strain RHΔku80Δhxgprt exhibited a detectable frequency of gene replacement using 230-bp

![Figure 3](image)

**FIG. 3.** Targeted gene replacement at the uracil phosphoribosyltransferase (UPRT) locus. (A) Strategy for disruption of UPRT by a double-crossover homologous recombination event in strain RHΔhxgprt or RHΔku80Δhxgprt by using a fixed 5′ target flank of 1.3 kb and a 3′ target DNA flank of 0.67 kb on plasmid pΔUPT-HXB. The PCR strategy for genotype verification is depicted using primer pairs to assay for products from the PCR (not to scale). (B) PFU assays were performed at various times after transfection to determine the GRF based on the fraction of parasites that were resistant to MPA (Table 2). (C) Genotype verification of clones selected for MPA resistance after transfection with the pΔUPT-HXB plasmid.
target DNA flanks, the overall efficiency was reduced by 22-fold compared to target DNA flanks of 450 bp or more (Fig. 5B). No gene replacement events were detected using target DNA flanks of 120 bp or less in any strain.

The efficiency of gene replacement at the HXGPRT locus was dependent on targeting DNA concentration (Fig. 5C). No gene replacement events were detected at the HXGPRT locus using circular targeting DNA in strain RH/hxgprt. In contrast, a detectable frequency was observed in strain RH/ku80/hxgprt, although the efficiency of gene replacement was reduced by 20-fold compared to linearized targeting DNA.

**DISCUSSION**

A nonreverting gene knockout at the KU80 locus functionally disrupts the NHEJ DSB DNA repair pathway in *T. gondii.*
Due to disruption of nonhomologous recombination mediated by the NHEJ pathway, KU80 knockouts exhibit a markedly higher gene replacement efficiency compared to wild-type strains. Our results suggest that disruption of KU80 leads to a nearly complete disruption of all nonhomologous recombination-mediated DSB DNA repair pathways in T. gondii. To our knowledge, this is the first report that clearly demonstrates the existence of a functional and significant KU-dependent NHEJ pathway in a protozoan parasite.

Readily identifiable KU genes are notably absent in Plasmodium species (http://plasmodb.org/plasmo; release 5). KU proteins are present but they do not participate in NHEJ in other protozoans such as Leishmania and Trypanosoma species (1, 24). It is puzzling that while NHEJ is prevalent in eukaryotes, a functional NHEJ pathway has not been previously described in protozoa. We speculate that acquisition and retention of a functional NHEJ DSB DNA repair pathway in T. gondii may correlate with the presence of extracellular developmental stages that occur in the oocyst. The oocyst stage must maintain viability through development and maintenance of sporocysts and sporozoites under harsh environmental conditions in soil and water for a long period of time prior to their successful transmission to intermediate hosts via oral ingestion.

The mechanisms of NHEJ had not been previously dissected in T. gondii. Our results show KU80 to be an essential component of the NHEJ mechanism in T. gondii. The Toxoplasma gondii genome (http://beta.toxodb.org/toxo5.0/) also reveals genes encoding putative DNA ligase IV (TGGT1_073840) and DNA-dependent protein kinase (57.m01765) components of eukaryotic NHEJ. Similar to other described proteins from parasites in the phylum Apicomplexa (16, 19), the predicted KU70 and KU80 proteins are markedly enlarged compared to other species of known KU proteins. Before initiating our studies at the KU80 gene, we targeted knockouts directed at both the KU70 gene and the DNA ligase IV gene, and these knockout attempts were not successful in strain RHΔhxgprt. Subsequent attempts to disrupt KU70 and the DNA ligase IV gene in the RHΔku80Δhxgprt background were also unsuccessful. Our negative results at these two loci suggest that these genes encode essential functions, or that their loci are refractory to gene targeting (data not shown).

KU80 knockouts retain a normal tachyzoite growth rate as well as high virulence typical of type I strains in murine infection. KU80 knockouts are highly stable in culture and have shown no fluctuation in growth rate, virulence, or the enhanced gene targeting phenotype after continuous passage for more than 1,600 generations. KU80 knockouts do exhibit an increased sensitivity to double-strand DNA breaks induced by phleomycin or γ-irradiation. No other major cellular or developmental defects have been noted so far in most fungal organisms disrupted in NHEJ (25, 35, 39, 41).

We find circular DNA to be a particularly poor substrate for double-crossover homologous recombination necessary for gene replacement in T. gondii. This conclusion is also supported by previous evidence (8). We find that DNA target flanks of 120 bp or less do not produce detectable gene replacements at the HXGPR1 locus. Using targeting strategies directed at the UPRT, CPSII, KU80, and HXGPR1 loci, we showed that efficient gene targeting now occurs in KU80 knockouts using target DNA flanks of ~500 bp or greater.

Recently, downstream markers such as UPRT, HXGPR1, or YFP have been used to enrich selected populations for desired double gene replacement events (23, 36, 37). Our results validate the use of the CD marker outside of the targeting cassette as another potent strategy to enrich for desired gene replacements in negative selection.

The HXGPR1 selectable marker was precisely “cleaned” from two loci (Δku80::HXGPR1 and Δuprt::HXGPR1) in two of the four sequential gene replacement steps used to develop the triple mutant strain RHΔku80ΔuprtΔhxgprt (Table 1). Strain RHΔku80Δhxgprt provides an excellent genetic background for the efficient development of multiply manipulated strains using only the HXGPR1 selectable marker.

Our results at the CPSII locus validate an efficient approach to complement a null phenotype (15) by direct gene replacement (of at least 24 kb) in strain RHΔku80Δhxgprt. This experiment illustrates that the RHΔku80Δhxgprt strain now enables more detailed studies on complementation and deciphering gene function(s) by directly targeting endogenous gene loci. This same knock-in double-crossover strategy can be used in the RHΔku80Δhxgprt strain to directly and efficiently tag a protein (c-myc, HA, YFP/GFP/eRF, etc.) or to rapidly place a gene under regulatable protein (28) or transcriptional (48) expression.

Parasites from the phylum Apicomplexa, including Plasmodium, Cryptosporidium, Babesia, Theileria, and other related species (Eimeria and Neospora) possess many genes that share significant homology with T. gondii genes. A significant fraction of these genes are also selectively unique to the Apicomplexa, and these genes are often designated as a “hypothetical protein” (www.EuPathDB.org). Consequently, any gene knockout developed in the Δku80 Δhxgprt genetic background that reveals a biological phenotype can be used in complementation studies to replace the inserted HXGPR1 marker (6TX negative selection) with the coding region of the complementing gene to clearly demonstrate a biological function for that “hypothetical” gene across the Apicomplexa.

Our results demonstrate that the KU80 knockout genetic background is a valuable tool for higher-throughput development of nonreverting gene knockouts and gene replacements necessary for postgenome functional analysis of T. gondii. Our laboratory developed the Δku80 Δhxgprt genetic background to enable a global genetic dissection of the Toxoplasma gondii “nutriome,” the collection of pathways and mechanisms controlling the acquisition of essential nutrients fueling obligate intracellular parasitism (2, 15, 17, 18, 20, 40). Fundamental questions remain to be answered as to how this clever obligate intracellular parasite has learned to successfully adapt to a large menu of host cells and hosts. The ability to now efficiently target gene replacements in the Δku80 background is an important advancement for this model organism. Accelerated genetic dissection of protozoan parasite biology will more quickly lead to new treatments for significant parasitic diseases.

ACKNOWLEDGMENTS

We thank Leah M. Rommereim (Dartmouth) for critical reading of the manuscript. We thank Nicholas C. Callahan for construction of several pHXH plasmids used in this study. The work of the developers...
of the Toxoplasma gondii Genome Information resource at www.ToxoDB.org is gratefully acknowledged.

This work was partially supported by the NIH (AI073142, AI07931, and AI41930). This work was supported by the use of facilities that were provided from the Irradiation Shared Resource of the Dartmouth-Hitchcock Norris Cotton Cancer Center. ToxoDB, PlasmoDB, and EuPathDB are part of the NIH NIAID-funded Bioinformatics Resource Center.

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