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Establishment of an *in vitro* Transgene Expression System in Epimastigotes of *Trypanosoma congolense*

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Abstract

The epimastigote form (EMF) of *Trypanosoma congolense* appears in the late tsetse infective stage. Epimastigotes adhere to the tsetse proboscis, proliferate in this region, and differentiate into mammal-infective metacyclic forms (MCFs). This differentiation is called metacyclogenesis and is indispensable for cyclical transmission of the parasite. Although an *in vitro* culture method reproducing metacyclogenesis was established several decades ago, few genetic tools have been utilized to elucidate the molecular mechanisms underlying *T. congolense* metacyclogenesis. In this study, we established a transgene expression system in the EMF of *T. congolense* IL3000: the EMF was successfully cultured and observed to undergo metacyclogenesis *in vitro*. The newly constructed expression vector pSAK was designed for integration into the repetitive α-β tubulin locus of the *T. congolense* genome. pSAK/enhanced green fluorescent protein (eGFP) was transfected into the EMF and procyclic form (PCF), which were cultured *in vitro* by electroporation. Both EMFs and PCFs expressing eGFP were successfully generated. The eGFP expressing EMFs differentiated into MCFs that continued to express eGFP. The *in vitro* transgenic EMF generation method is expected to contribute to the elucidation of molecular mechanisms underlying metacyclogenesis.

Keywords

*Trypanosoma congolense*; epimastigotes; *in vitro* metacyclogenesis; transfection; transgene expression

*Trypanosoma congolense* is a hemoflagellate protozoan parasite responsible for animal African trypanosomosis (nagana) in sub-Saharan Africa. This parasite is primarily transmitted by the tsetse fly (*Glossina* spp.) and undergoes drastic cell differentiations in order to adjust to various different environments. Briefly, the bloodstream form (BSF) parasitizes blood circulation of the mammalian host and is ingested by the tsetse when it sucks the host’s blood. In the tsetse midgut, BSF differentiates into the procyclic form (PCF) that migrates to the proboscis of the tsetse and further differentiates into epimastigote form (EMF) [1]. The EMFs adhere to the substrate of the tsetse proboscis and differentiate into metacyclic forms (MCFs), which reinfect the mammalian host and differentiate into BSFs. The differentiation (EMF to
MCF) is called metacyclogenesis and indispensable for cyclical transmission of the parasite [2, 3].

In trypanosomes, transgene expression systems are powerful tools to study the biological functions of the parasite proteins. In the T. brucei subspecies, inducible transgene expression systems have been well established [4]. In particular, knocking down the expression of certain genes by double-stranded RNA interference (dsRNAi) has contributed greatly to the biology of these parasites [5]. While these systems have been extensively used for studies on the BSF and PCF, they have not been widely used for research on the EMF. EMFs of only a few African trypanosome isolates have successfully been cultured in vitro, while culture systems for the BSFs and PCFs are well established. Although the EMF is found in experimentally infected tsetse, it is very difficult to establish genetically manipulated EMF cell lines. One African trypanosome isolate, the EMF of T. congolense IL3000 can be grown, and it can differentiate into the MCF in vitro (in vitro metacyclogenesis system). Foreign protein expression in the PCF of T. congolense has been previously reported by the same methodology as that used in the case of T. brucei [6]. In this study, we established a foreign-gene overexpression system in the T. congolense EMF by using the in vitro metacyclogenesis system and the newly constructed expression vector pSAK. The generated eGFP-expressing EMF (EMF-eGFP) differentiates into the MCF, which also express eGFP. This is the first report showing the generation of transgenic EMFs and MCFs in vitro.

At the beginning of this study, we constructed a novel foreign protein expression vector for T. congolense and named it pSAK. The anatomy and target locus of pSAK are shown in Fig. 1. Briefly, pSAK was designed for integration into the repetitive α-β tubulin locus of the T. congolense genome (predicted with T. congolense sequence data obtained from the Sanger Institute website at http://www.sanger.ac.uk/Projects/T_congolense/). The enhanced green fluorescence protein (egfp) gene and hygromycin B phosphotransferase (hyg) gene were used as a reporter and drug selectable marker, respectively. The all regions of the pSAK expression cassette were polymerase chain reaction (PCR)-amplified using the parasite's genomic DNA as a template and the following primers (restriction sites underlined). The upstream region (719 bp) of β-tubulin containing a part of the α-tubulin coding region (362 bp) followed by an intercoding region (ICR) (357 bp) was amplified with the primer set for fragment A (5′-GGG CCC TCG CGA CCA TCA AGA CGA AG-3′ and 5′-AAG CTT GAT GGA ATT GGA TTA GT CTT-3′). While the β-tubulin downstream region (617 bp) containing a part of α-tubulin coding region (174 bp)
following an ICR (443 bp) was amplified with the primer set for fragment B (5’-GAG
CTC AGC GCA GTT GCC GAC GAA TC-3’ and 5’-GAG CTC GGC GCC AGT CTC AGA
GAA GA-3’). The ICR (259 bp) between 2 tandem P0 (ribosomal subunit protein) genes
was amplified with the primer set for P0-ICR (5’-GGA TCC TTG ATT TCT TTT CTA
ATT TT-3’ and 5’-GGA TCC AAA TGT AAA CTA TGG AGG TT-3’). egfp and hyg were
amplified from pEGFP (Clontech Laboratories Inc., Mountain View, CA, U.S.A.) and
pLEW 29 (gift from Dr. J. E. Donelson, Iowa University) [4] by using primers for egfp
(5’-AAG CTT ATG GTG AGC AAG GGC GAG GA-3’ and 5’-AAG CTT TTA CTT GTA
CAG CTC CGC AC-3’) and hyg (5’-TCT AGA ATG AAA AAG CCT GAA CTC AC-3’ and
5’-TCA GTT AGC CTC CCC CAT CT-3’), respectively. All PCR products were cloned
once into pCR-Blunt II-TOPO (ZERO Blunt TOPO PCR Cloning Kit, Invitrogen,
Carlsbad, CA, U.S.A.), digested with optimal restriction enzymes (BamHI for P0-ICR,
SacI for fragment B, XbaI for hyg (3’ restriction site present within pCR-Blunt II-TOPO),
ApaI and HindIII for fragment B, and HindIII for egfp) and sequentially subcloned into
the pBluescriptII SK+ cloning vector (Novagen, Darmstadt, Germany). The
pSAK/eGFP (50 µg) was digested with NarI and NruI (restriction sites within the
primers are represented by the italicized letters), purified, and dissolved in 50 µl
distilled water prior to transfection.

Then, parasites were transfected with pSAK. All the parasites used in this
study were those specific to the T. congolense IL3000 strain, which is a savanna-type
parasite. The EMFs and PCFs have been maintained in vitro in the TVM-1 medium as
described previously [7, 8]. Since the transfection procedure of T. congolense PCF was
already established, pSAK/eGFP was transfected into the PCF in order to examine the
expression efficacy of pSAK, as reported previously [6, 9]. The PCF was transferred
into 10 ml of fresh TVM-1 medium immediately after the electroporation and incubated
at 27 °C for 24 h prior to adding hygromycin B (Wako Pure Chemical Industries Ltd.,
Osaka, Japan). It is known that PCF of T. congolense does not grow if diluted below
10^5 cells/ml [6]. Therefore, the transfected PCF was first maintained with a nonlethal
dose of hygromycin B (12.5 µg/ml) for a week along with regular addition of fresh TVM-1
containing hygromycin B. After confirming the proliferation and accumulation of
eGFP-expressing PCF, the concentration of hygromycin B was elevated to 50 or 100
µg/ml in order to eliminate wild-type parasites within a week. Thereafter, the
PCF-eGFP was maintained under similar conditions as the wild-type PCF. The
non-fixed PCF-eGFP was observed by confocal laser scanning microscopy (TCS-NT,
Leica Microsystems GmbH, Wetzlar, Germany) as shown in Fig. 2, panels A–C. This
result showed that pSAK can drive gene expression in T. congolense. Subsequently,
pSAK/eGFP was also transfected into the EMF. In order to avoid the mechanically damaged parasites, naturally detached EMFs in the confluent culture supernatant were collected, washed, and subjected to transfection by the same method as that followed for the PCF [6, 9]. Hygromycin B was added to the parasite culture at a concentration of 12.5 µg/ml for 24 h after the transfection. Wild-type EMF was eliminated within a week, while the surviving transfectant adhered to the plastic surface, proliferated, and formed an equal number of colonies as that of the wild type EMF (Fig. 2, panels D–F). The EMF-eGFP has been maintained under the same conditions as that of the wild type EMF, as recently reported [7, 8]. The MCF derived from EMF-eGFP was separated from the culture supernatant by the use of DE52 anion-exchange column chromatography (Whatman plc., Brentford, U.K.) as described previously [10]. Confocal laser scanning microscopic observations showed that the MCF continued to express eGFP (Fig. 2, panels G–I). The integration of pSAK into the target locus of the parasite genome was confirmed by PCR using the genomic DNA extracted from the EMF and PCF as templates (data not shown). The expression of egfp and hyg in the EMF, MCF, and PCF were confirmed by reverse transcription PCR (RT-PCR) (data not shown). The EMF-eGFP and PCF-eGFP that were maintained for 6 months without hygromycin B stably expressed eGFP.

This is the first report of in vitro transgenic EMF generation. It was confirmed that the novel expression vector pSAK integrated into the parasite's genome and stably functioned in the EMF and PCF. Though the same methodology of the expression-vector transfection was applied to the PCF and EMF, a higher dose of hygromycin B was required for the selection of the transgenic PCF compared to EMF. An important point to be noted in this study is that the generated EMF-eGFP was capable of undergoing metacyclogenesis into the MCF, which continued to expresses eGFP. This result indicates that pSAK-transfection itself does not interfere with metacyclogenesis and the expressions are carried out from the EMF to MCF. Although more useful systems such as inducible overexpression and dsRNAi optimized for T. congolense are desirable [9], we expect that our system will contribute to molecular biological approaches for revealing the mechanisms underlying metacyclogenesis.

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sequence data was obtained from the Sanger Institute website at http://www.sanger.ac.uk/Projects/T_congolense/. Sequencing of the T. congolescense genome was funded by the Wellcome Trust. This study was supported by a Grant-in-Aid for scientific Research from JSPS to SK and NI.

References


Figure legends

Fig. 1. Anatomy and target locus of pSAK. The expression cassette of pSAK was designed for integration into the α-β tubulin locus of the T. congolescense genome. Bars A and B represent fragments A and B respectively.
Fig. 2. Confocal laser scanning microscope images of eGFP-expressing parasites. Panels A–C, PCF-eGFP; panels D–F, EMF-eGFP forming colonies; panels G–I, MCF-eGFP purified from the EMF culture supernatant. Non-fixed parasites were subjected to observations.
Fig. 2. Sakurai et. al.