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

Tatsuya Sakurai, Miho Tanaka, Shin-ichiro Kawazu, Noboru Inoue*

National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Nishi 2-13, Inada-cho, Obihiro, Hokkaido 080-8555, Japan

*Corresponding Author:
Dr. Noboru Inoue, D.V.M., Ph.D.
National Research Center for Protozoan Diseases,
Obihiro University of Agriculture and Veterinary Medicine
Nishi 2-13, Inada-cho, Obihiro, Hokkaido 080-8555, Japan
Tel: +81-155-495647; Fax: +81-155-495643; E-mail: ircpmi@obihiro.ac.jp

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′-<u>GGG CCC</u> *TCG CGA* CCA TCA AGA CGA AG-3′ and 5′-<u>AAG CTT</u> 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 ACG 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 GTC CA-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 (*Bam*HI for P0-ICR, *Sac*I for fragment B, *Xba*I for *hyg* (3′ restriction site present within pCR-Blunt II-TOPO), *Apa*I and *Hin*dIII for fragment B, and *Hin*dIII for *egfp*) and sequentially subcloned into the pBluescriptII SK+ cloning vector (Novagen, Darmstadt, Germany). The pSAK/eGFP (50 μg) was digested with *Nar*I and *Nru*I (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.

Acknowledgements

The authors thank Dr. J. E. Donelson, Iowa University for providing the plasmid. The authors are grateful to Dr. Makoto Igarashi, Obihiro University of Agriculture and Veterinary Medicine for the valuable suggestions. *Trypanosoma*

congolense sequence data was obtained from the Sanger Institute website at http://www.sanger.ac.uk/Projects/T_congolense/. Sequencing of the *T. congolense* 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

[1] Prain CJ, Ross CA. *Trypanosoma congolense*: appearance and distribution of variable antigen types during metacyclic differentiation in vitro. Parasitology 1990;100 Pt 1:107-13.

[2] Vickerman K, Tetley L, Hendry KA, Turner CM. Biology of African trypanosomes in the tsetse fly. Biol Cell 1988;64: 109-19.

[3] Hendry KA, Vickerman K. The requirement for epimastigote attachment during division and metacyclogenesis in *Trypanosoma congolense*. Parasitol Res 1988;74: 403-8.

[4] Wirtz E, Leal S, Ochatt C, Cross GA. A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. Mol Biochem Parasitol 1999;99: 89-101.

[5] LaCount DJ, Donelson JE. RNA interference in African trypanosomes. Protist 2001;152: 103-11.

[6] Downey N, Donelson JE. Expression of foreign proteins in *Trypanosoma congolense*. Mol Biochem Parasitol 1999;104: 39-53.

[7] Hirumi H, Hirumi K. In vitro cultivation of *Trypanosoma congolense* bloodstream forms in the absence of feeder cell layers. Parasitology 1991;102 Pt 2: 225-36.

[8] Sakurai T, Sugimoto C, Inoue N. Identification and molecular characterization of a novel stage-specific surface protein of *Trypanosoma congolense* epimastigotes. Mol Biochem Parasitol (in press).

[9] Inoue N, Otsu K, Ferraro DM, Donelson JE. Tetracycline-regulated RNA interference in *Trypanosoma congolense*. Mol Biochem Parasitol 2002;120: 309-13.

[10] Lanham SM, Godfrey DG. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. Exp Parasitol 1970;28: 521-34.

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. congolense* 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.

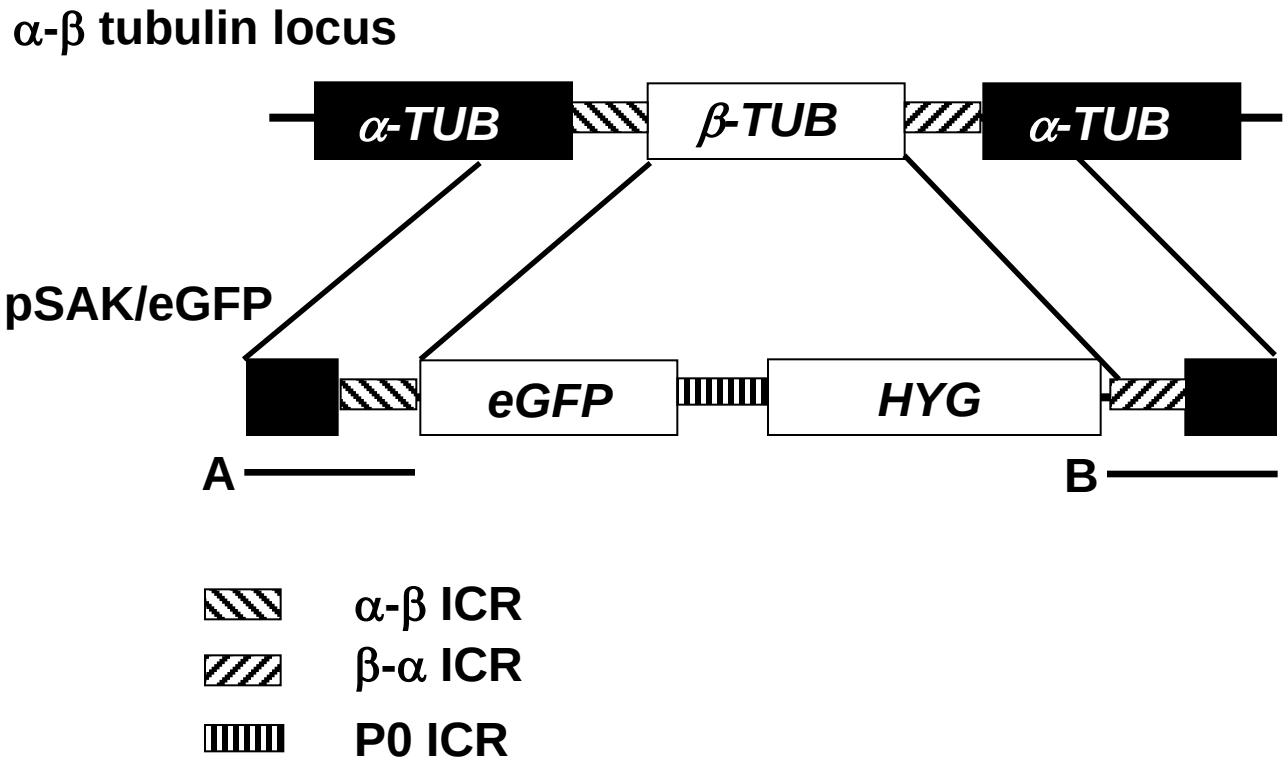

Fig. 1. Sakurai et. al.

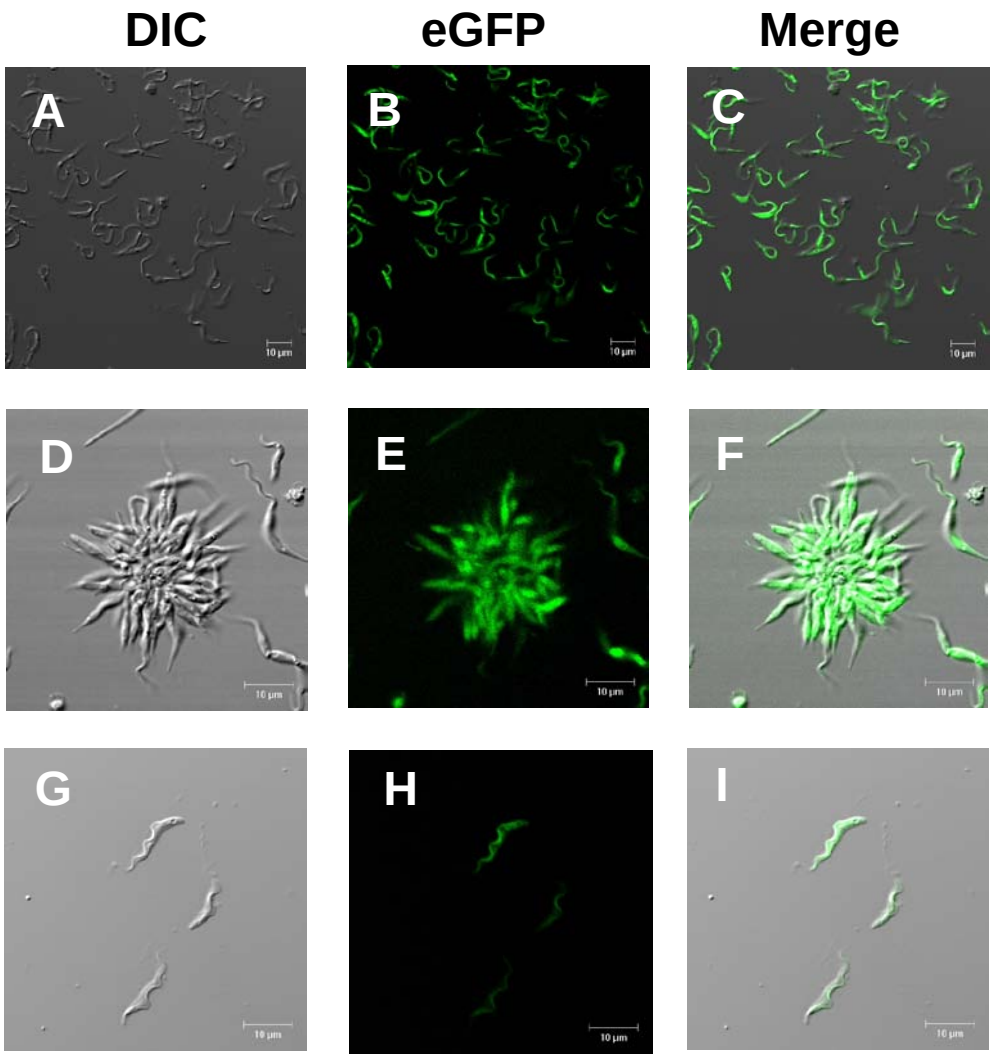

Fig. 2. Sakurai et. al.