Expression of Fluorescent Genes in *Trypanosoma cruzi* and *Trypanosoma rangeli* (Kinetoplastida: Trypanosomatidae): Its Application to Parasite-Vector Biology

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ABSTRACT Two *Trypanosoma cruzi*-derived cloning vectors, pTREX-n and pBs:CalB1/CUB01, were used to drive the expression of green fluorescent protein (GFP) and DsRed in *Trypanosoma rangeli* Tejera, 1920, and *Trypanosoma cruzi* Chagas, 1909, isolates, respectively. Regardless of the species, group, or strain, parasites harboring the transfected constructs as either episomes or stable chromosomal integrations showed high-level expression of fluorescent proteins. Tagged flagellates of both species were used to experimentally infect *Rhodnius prolixus* Stal, 1953. In infected bugs, single or mixed infections of *T. cruzi* and *T. rangeli* displayed the typical cycle of each species, with no apparent interspecies interactions. In addition, infection of kidney monkey cells (LLC-MK2) with GFP-*T. cruzi* showed that the parasite retained its fluorescent tag while carrying out its life cycle within cultured cells. The use of GFP-tagged parasites as a tool for biological studies in experimental hosts is discussed, as is the application of this method for copopulation studies of same-host parasites.

KEY WORDS *Trypanosoma cruzi*, *Trypanosoma rangeli*, green fluorescent protein, mixed infection

Trypanosoma cruzi Chagas, 1909, causes Chagas' disease, a debilitating and often incurable ailment affecting nearly 20 million people in endemic areas of South and Central America. In some of these areas, another protozoa, *Trypanosoma rangeli* Tejera, 1920, shares insect vectors and mammalian hosts with *T. cruzi* (D'Alessandro-Bacigalupo and Saravia 1992). Unlike *T. cruzi*, *T. rangeli* has pathological effects on the insect vector (Tobie 1965, Watkins 1971, Añez 1984), but it is harmless to the mammalian host (D'Alessandro-Bacigalupo and Saravia 1992). *T. rangeli* is transmitted to vertebrates by the bite of triatomine bugs (Tobie 1965, Añez 1984), whereas *T. cruzi* transmission occurs by fecal contamination. Mixed infections with both species of parasites in mammalian and triatomine hosts are not uncommon (Hoare 1972).

In laboratory experiments, the innocuous, strongly fluorescent green fluorescent protein (GFP) is an important tool for tagging cells. GFP expression does not require cofactors such as ATP or reduced coenzymes, and it has proved invaluable for the in vivo visualization of cell processes (Southward and Surette 2002). However, there have been few reports on the use of GFP for examining parasite–vector interactions (Bingle et al. 2001, Guevara et al. 2001). In the present work, the *T. cruzi* cloning vector pTREX-n (Vazquez and Levin 1999) was used to express GFP and DsRed in different *T. cruzi* and *T. rangeli* isolates. The resulting tagged parasites were used to examine the life cycles of *T. cruzi* and *T. rangeli* in the vector *Rhodnius prolixus* Stal, 1859. In addition, in vitro cultured kidney monkey cells were used to observe the invasion and intracellular multiplication of GFP-tagged *T. cruzi.*

Materials and Methods

Parasites. Venezuelan isolates of *T. cruzi* (MHOM/Ve/92/2-92-YBM and MHOM/Ve/91/1-91-JMP) and *T. rangeli* (MCAN/Ve/82/Dog-82, IRHO/Ve/98/Triat-1, IRHO/Ve/98/Triat-2, and MMAC/Ve/98/Mono) and a *T. cruzi* Brazilian reference strain (CL strain clone-Brener) (Cano et al. 1995) were used. Parasites were grown in Liver Infusion Tryptose (LIT) medium until they reached a density of 5×10^6 cells/ml. GFP- and DsRed-tagged *T. cruzi* and *T. rangeli* were cultured in NNN media supplemented with 500 μg/ml of geneticin G418 antibiotic.

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***T. cruzi* Cell Infection Assays.** Subconfluent cultures of LLC-MK2 cells were infected with metacyclic forms of *T. cruzi* isolate GFP-MHOM/Ve/92/2-92-YBM. After 48–72 h, free parasites were washed away and the infected LLC-MK2 cells were maintained in 2% fetal calf serum-RPMI-1640, at 37°C in 5% CO_2. Trypomastigotes (TCT) were obtained from cell supernatants and used for subsequent infection of new LLC-MK2 cultures.

Triatomine Bugs. Nymphs (fourth and fifth instar) of *R. prolixus* were reared in closed colonies for use in this work (Añez and East 1984).

Triatomine Experimental Infections. Cultured flagellates were collected by centrifugation at 4,000 × *g*. The supernatants were discarded and the pellets were resuspended in defibrinated rabbit blood to a concentration of 5×10^6 parasites per milliliter. This mixture was then placed in an artificial feeding system coupled to a circulating water bath adjusted to 37°C (Garcia et al. 1984). Batches of 25 bugs each were allowed to feed for 30 min. Engorged insects were kept at 25°C with 80% humidity and a photoperiod of 12:12 (L:D) h. Systematic observations were performed at zero hour, daily up to day 15, and every 7 d thereafter until day 31. Hemolymph was sampled from the cut end of one leg per infected bug, smeared on a glass slide, and examined by fluorescent and light microscopy. Other specimens were dissected at different times and parts of their digestive tracts were teased apart and placed on glass slides for observation. Fluorescence observations were performed on an Axioscope fluorescent microscope (Carl Zeiss, Jena, Germany) by using an excitation wavelength of 520 nm and observing with a filter with a range between 450 and 490 nm. In this way, a compromise emission wavelength was reached that allows the simultaneous observation of green and red. Photographs were taken with a fully automated MC-80 camera (Carl Zeiss). Because parasites were not fixed, their movement produced blurred images.

Fluorescence Level Determination. A total of 10^7 GFP-labeled parasites were adjusted to 500 μl in saline solution (0.85% NaCl), placed in quartz cuvettes, and analyzed in a fluorescence spectrophotometer (model F 2000, Hitachi, Tokyo, Japan) with excitation at 495 nm and detection at 515 nm. The background was set in comparison to the same concentration of nonfluorescent cells, and fluorescence was expressed as arbitrary units.

Typing of *T. cruzi* and *T. rangeli* Isolates. Before experimental infections, *T. cruzi* and *T. rangeli* isolates were typed. For typing *T. cruzi*, we used two species-specific polymerase chain reaction (PCR) assays: the first targeted repeated sequences of the intergenic ribosomal spacer (SER) (Novak et al. 1993, González et al. 1994), and the second targeted the C6-interspersed repetitive DNA element (Araya et al. 1997). *T. rangeli* isolates were identified with a species-specific PCR assay directed to the P542 repetitive element (Vargas et al. 2000). All amplification reactions were carried out on 10 ng of genomic DNA by using the primers, reaction conditions and amplification parameters described in the original publications.

***T. cruzi* rDNA Group Typing.** For this purpose, we examined a "group-specific" PCR fragment found in the 24S subunit ribosomal gene, as described previously by Souto and Zingales (1993).

Parasite DNA Isolation. Cultured parasites were harvested at a cell density of 5×10^6 flagellates per milliliter and lysed by incubation in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5% SDS, 1 mM EDTA, followed by digestion with proteinase K (2 μg/ml). DNA was isolated by phenol:chloroform extraction, and total nucleic acids were recovered by ethanol precipitation. The DNA of *T. rangeli* strain San Agustin was generously donated by Dr. John Swindle (Infectious Disease Research Institute, Seattle, WA).

GFP and DsRed Plasmid Constructs. A *Hind*III/*Xho*I fragment derived from pGFP5(S65T) containing the mgfp5(S65T) gene version of GFP (Siemering et al. 1996) was cloned into pTREX-n digested with *Hind*III/*Xho*I (Fig. 2A), resulting in plasmid pTREXn-GFP5(S65T). The DsRed construct was made by replacing the GFP gene in pTREXn-GFP5 (S65T) with an *Eco*RI/*Not* fragment derived from plasmid pDsRed1.1 (BD Biosciences Clontech, Palo Alto, CA). The final construct was named pTREXn-DsRed1.1 (Fig. 2A). pBsCalB1/CUB01 constructs expressing GFP were obtained by replacing the CUB gene with an *Xba*I fragment derived from pEGFP, containing the EGFP version of GFP (BD Biosciences Clontech) (Fig. 4B). The resulting construct, pBs:CalB1/CUB01-EGFP, was converted to the DsRed vector by replacement of the EGFP gene with an *Xma*I/*Not*I fragment derived from plasmid pDsRed1.1 (Fig. 4B).

Parasite Transfections and Selection of *T. cruzi* and *T. rangeli* Stable Fluorescent Cell Lines In Vivo. The electroporation protocol used for *T. cruzi* and *T. rangeli* was as described by Hariharan et al. (1993). Cultured flagellates were grown to mid-log phase, harvested by centrifugation, and washed with LIT media minus hemin and serum (LIT-HS). Cells were adjusted with LIT-HS to a final concentration of 8.5×10^8/ml, 200 μg of plasmid DNA was added to 0.35 ml of cell suspension in a 2-mm gap electroporation cuvette (BTX), and the mixtures were incubated at 4°C for 10 min. Cells were transfected by a single electric pulse of 300 V, 1000 μF, and 100 Ω by using a Gene Pulser II (Bio-Rad, Hercules, CA). Electroporated cells were resuspended in 10 ml of complete LIT medium and incubated at 28°C. Forty-eight hours later, 500 μg/ml of G418 was added to the media. After 15 d, the antibiotic was withdrawn.

Chromosomal Band Analysis. Pulse field gel electrophoresis (PFGE) of transformed *T. rangeli* cell lines was performed on a CHEF-DR III System apparatus (Bio-Rad). Agarose blocks were prepared as described previously (Galindo and Ramírez 1989). A positive control agarose block was prepared with wild-type *T. rangeli* cells and 0.1 μg of pTREX-GFP5(S65T) DNA. For PFGE, a 1% agarose gel was run at 14°C in 0.5× Tris borate-EDTA buffer at 6 V/cm at 120° sep-

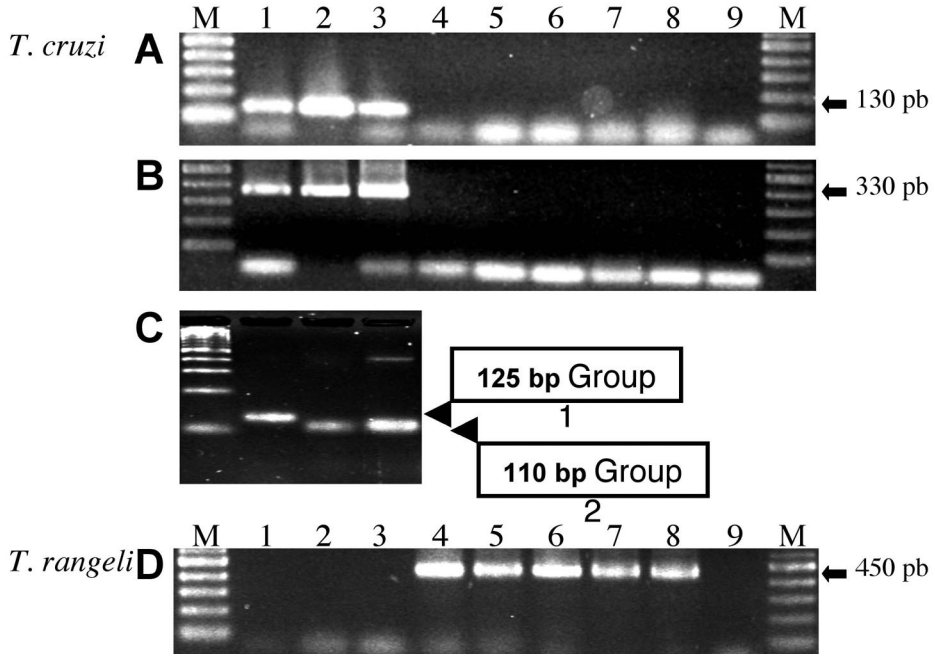

Fig. 1. *T. cruzi* and *T. rangeli* PCR species identification and rDNA group typing. (A and B) *T. cruzi* species were identified by PCR assays for repeated SER sequences and the C6 interspersed repetitive DNA element respectively. (C) *T. cruzi* rDNA group typing by amplification of the 24S subunit ribosomal gene. (D) *T. rangeli* species-specific PCR assays directed to the P542 repetitive element. Lanes: M, 100-bp ladder; 1, *T. cruzi* CL Brener; 2, *T. cruzi* JMP; 3, *T. cruzi* YBM; 4, *T. rangeli* Triat-1; 5, *T. rangeli* Dog-82; 6, *T. rangeli* San Agustín; 7, *T. rangeli* Mono; 8, *T. rangeli* Triat-2; and 9, H_2O.

aration angle with 60–120-s switching time for 20 h, followed by 200–200-s switching time for 14 h at 4.5 V/cm, and 240–240-s switching time for 6 h at 4 V/cm. The gel was stained with ethidium bromide (0.5 μg/ml) and visualized in a UV transilluminator. Gels were then blotted onto Hybond-N membranes (Amersham Biosciences UK, Ltd., Paisley, UK) by capillary action for 24 h, UV cross-linked, hybridized, washed, and autoradiographed, all using standard protocols (Sambrook et al. 1989).

DNA Probes. To obtain the GFP probe, plasmid pTREXn-GFP was digested with *Bam*HI/*Not*I, and an 800-base pair (bp) fragment was agarose gel purified (Fig. 2A). The DsRed probe was isolated from *Eco*RI/*Not*I digested pTREXn-DsRed (Fig. 2A) in the same manner. The 26S rDNA probe was derived from a 1.2 kb *Hind*II/*Xho*I fragment cloned into pLma18.2 (P.G., unpublished data). The *T. rangeli* GADPH 1-kb coding sequence was amplified from *T. rangeli* Triat-1 genomic DNA by using primers GAPDH cod5′ F: 5′ CCC ATC AAG GTC GGY ATC AAC GGC 3′ and GAPDH cod3′ R: 5′ AGG TCC ACC ACG CGG TGS GAG TA 3′, which were derived from consensus sequences from the reported *T. cruzi* and *Leishmania mexicana* Biagi, 1953, genes (Kendall et al. 1990, Hannaert et al. 1992). All probes were random primer labeled with [α-^{32}P]dCTP by using standard protocols (Sambrook et al. 1989).

Results

***T. rangeli* and *T. cruzi* Identification and Typing.** Amplification of DNA from *T. cruzi* isolates with species-specific PCR primers yielded the expected amplification products for the SER (130 bp; Fig. 1A) and C6 interspersed repeated sequences (330 bp; Fig. 1B). Also, the two *T. cruzi* Venezuelan isolates (JMP and YBM) yielded the typical 110-bp group-2 ribotype amplification band, whereas the CL Brener isolate yielded the expected group-1 125-bp amplification product (Fig. 1C). All isolates were then tested with a *T. rangeli* species-specific PCR assay. As shown in Fig. 1D, only *T. rangeli* cultures showed positive amplification of the expected 450-bp product.

***T. rangeli* and *T. cruzi* Transfection Experiments.** Fig. 2A shows the pTREX-n-based constructs used for the transfection experiments. This vector contains the ribosomal promoter (RP) of group-2 *T. cruzi* strain La Cruz (Martinez-Calvillo 1998), followed by an HX1 transplicing region derived from the *T. cruzi* ribosomal protein TcP2β gene (Vazquez and Levin 1999). The genes for GFP [version mgfp5(S65T)] or DsRed 1.1 were inserted downstream of the RP. In pTREX-n, the inserted marker genes were followed by the antibiotic marker (NEO) and *T. cruzi* GADPH intergenic sequences. Figure 2B shows a mix of GFP-*T. rangeli* and DsRed-*T. cruzi*-tagged cells. Fluorescence was evenly spread throughout the cell body, including the flagella.

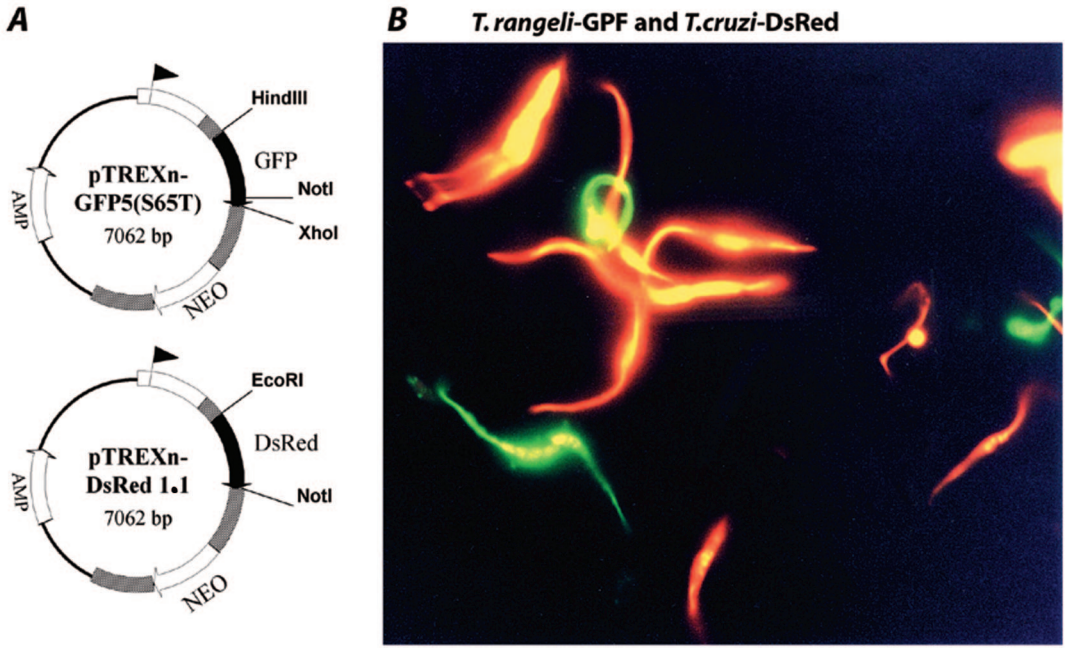

Fig. 2. Stable expression of fluorescent markers driven by the RP. (A) Map of GFP and DsRed pTREX-n constructs. The white bar and the black flag mark the RP and the transcriptional start point, respectively. Black bars with arrows indicate the GFP and DsRed genes and the direction of transcription. Dashed bars show intergenic regions. White bars with arrows show the positions of the ampicillin and neomycin resistance genes. (B) DsRed-*T. cruzi* and GFP-*T. rangeli*-tagged epimastigotes in mixed cultures. Magnification, 400×.

Figure 3 shows the time course of GFP expression in *T. rangeli* and *T. cruzi* cells transfected with pTREXn-GFP. At day 1, without G418, 5 to 10% of cells showed a strong transient expression of the green marker. Once the antibiotic was added at day 2, the number of green fluorescent cells steadily increased, reaching 100% at days 7 and 10 for *T. cruzi* and *T. rangeli*, respectively. The antibiotic was removed 15 d post-electroporation, and there was no evident decrease in the number of fluorescent cells, indicating that the GFP was stably integrated into the chromosome. All strains transfected with pTREX-n vectors retained their fluorescence levels after 1 yr of culturing without antibiotic selection (Table 1).

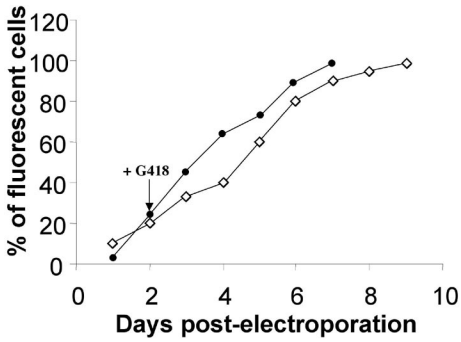

Fig. 3. *T. rangeli* and *T. cruzi* expression of RP-driven GFP over time. *T. rangeli* Triat-1 is indicated by empty diamonds and *T. cruzi* CL Brener is denoted by filled circles.

The second type of constructs was based on vector pBs:CalB1/CUB01 (Swindle, unpublished data). These included *T. cruzi* ubiquitin transcription regulatory and transplicing sequences from the calmodulin-ubiquitin 2.65 locus (Ajioka and Swindle 1993), which had been previously tested in *T. cruzi* expression vectors (Laurent and Swindle 1999) (Fig. 4A). Although the *T. rangeli* calmodulin-ubiquitin locus has a similar gene organization to that of *T. cruzi*, there is no known sequence homology in the intergenic regions (J. Swindle, personal communication). Unlike the pTREXn-GFP vectors, the pBs:CalB1/CUB01-derived plasmids required G418 for stability and continuous expression of the fluorescent markers (Table 1).

Chromosomal Integration of GFP Genes. The localization of the GFP genes in transfected cells was determined by Southern blot analysis of PFGE-resolved chromosomal bands hybridized with a radiolabeled GFP probe. In *T. cruzi* cells transfected with pTREXn-GFP, the probe recognized unique chromosomal bands that ranged in size from 1.1 to 2.7 Mbp (according to the strain). These results coincided with the location of the ribosomal gene locus in that strain (Table 1; data not shown). A similar analysis for *T. rangeli* recognized a 0.915-Mbp band (Fig. 5, lanes 6–11), and in some cases a second band of 0.945 Mbp (Fig. 5, lanes 8 and 9). Neither of these matched the expected two Mbp ribosomal band. To test whether

Table 1. *T. cruzi* and *T. rangeli* analysis of transfected strains

Strain	Expression vector	Fluorescent marker	Expression	Fluorescence levels[a]	Chromosomal location
T. cruzi CL Brener	pTREX-n	GFP5(S65T)	Stable	5.6	1.6 Mbp
T. cruzi JMP	pTREX-n	GFP5(S65T)	Stable	14.2	1.1 Mbp
T. cruzi YBM	pTREX-n	GFP5(S65T)	Stable	36.2	ND
T. rangeli Triat-1	pTREX-n	GFP5(S65T)	Stable	16.2	0.915, 0945 Mbp and extrachromosomal
T. rangeli Dog-82	pTREX-n	GFP5(S65T)	Stable	10.8	ND
T. cruzi CL Brener	pBs:CalB1/CUB01	EGFP	G418 required	0.7	2.7 Mbp and extrachromosomal
T. cruzi CL Brener	pBs:CalB1/CUB01	DsRed 1.1	G418 required	0.8	2.7 Mbp and extrachromosomal
T. cruzi JMP	pBs:CalB1/CUB01	EGFP	G418 required	0.6	Extrachromosomal
T. cruzi JMP	pBs:CalB1/CUB01	DsRed 1.1	G418 required	0.6	Extrachromosomal
T. cruzi YBM	pBs:CalB1/CUB01	EGFP	G418 required	0.7	Extrachromosomal
T. cruzi YBM	pBs:CalB1/CUB01	DsRed 1.1	G418 required	0.9	Extrachromosomal
T. rangeli Triat-1	pBs:CalB1/CUB01	EGFP	G418 required	3.3	Extrachromosomal
T. rangeli Triat-1	pBs:CalB1/CUB01	DsRed 1.1	G418 required	1.2	Extrachromosomal
T. rangeli Dog-82	pBs:CalB1/CUB01	EGFP	G418 required	2.2	ND
T. rangeli Dog-82	pBs:CalB1/CUB01	DsRed 1.1	G418 required	1.1	ND

ND, not determined.
[a] Arbitrary units.

integration had occurred at the GADPH locus, we hybridized *T. rangeli* PFGE blots with a probe consisting of the coding region of the *T. rangeli* GADPH gene. The *T. rangeli* GADPH gene was thus localized to a 1.8-Mbp band (data not shown), which was inconsistent with the localization of the integrated construct. Our inability to concretely establish the integration point of the GFP gene in *T. rangeli* was possibly due to the existence of regions homologous to the *T. cruzi* RP elsewhere in the genome.

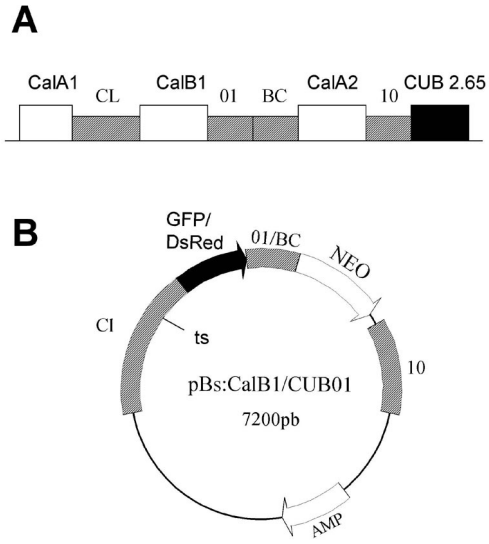

Fig. 4. Expression vector based on sequences in the calmodulin/ubiquitin locus of *T. cruzi.* (A) Partial map of *T. cruzi* calmodulin/ubiquitin 2.65 locus. (B) Map of the pBS:CalB1/CUB01-EGFP expression construct. Intergenic untranslated sequences in the 2.65 calmodulin/ubiquitin locus and in the expression vector are indicated by dashed bars. Coding genes are represented by white boxes. Black bars with arrows indicate EGFP or DsRed genes and direction of transcription. White bars with arrows show the position of the ampicillin and neomycin resistance genes. Transplicing site, ts.

Except for CL Brener cells transfected with pBs:CalB1/CUB01EGFP, all experiments using the pBs:CalB1/CUB01 vector were unstable, with cells requiring continued antibiotic selection to maintain GFP expression (Table 1). Because experiments in bugs demand an absence of antibiotics, this construct was not used further.

Life Cycles of Tagged *T. cruzi* and *T. rangeli* in *R. prolixus*. Fig. 6 summarizes the life cycle of GFP-*T. rangeli* in infected *R. prolixus.* Details on the time of development and the distribution of different forms of the parasite in various parts of the insect's body are given in the figure. Similar to what is observed in nontagged *T. rangeli*, GFP parasites were evident in the digestive tract, the hemolymph and the salivary glands of *R. prolixus* (Fig. 6B, C, D, F, and insert), and in the insect feces (data not shown). In mixed infections, GFP-*T. rangeli* and DsRed-*T. cruzi* flagellates could be observed in the bug's gut from 7 to 20 d postinfection (Fig. 7B and C). In coinfected bugs, invasion into the hemolymph was only observed with GFP-*T. rangeli.* In these cases, both extracellular development in the hemolymph and intrahemocytic invasion by GFP-*T. rangeli* was detected at day 21 postinfection (Fig. 7D and E).

As in the case of single infections by *T. rangeli*, 1 wk after the parasites reached the hemolymph, slender GFP-flagellates and GFP-metacyclic forms could be observed inside the salivary glands (data not shown). Although green and red parasites were detected in the insect hindgut, only *T. cruzi* red cells displayed the typical trypomastigote infective forms (data not shown).

***T. cruzi* In Vitro Intracellular Cycle.** As shown in Fig. 8, *T. cruzi* YBM-GFP trypomastigotes released

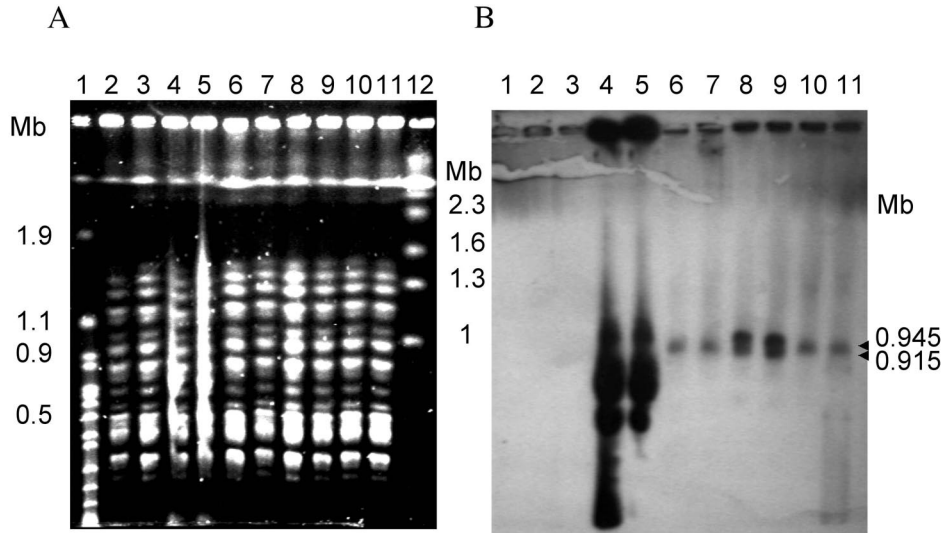

Fig. 5. Genomic localization of GFP. (A) Gel of chromosomal bands from control and transfected *T. rangeli* cell lines. (B) Southern blot of A with GFP-specific probe. The arrows indicate the chromosomal bands identified by the probe. Lanes are 1, *Saccharomyces cerevisiae* chromosomes; 2 and 3, *T. rangeli* Triat-1 wild type; 4 and 5, *T. rangeli* Triat-1 wild type + plasmid pTREXn-GFP5(S65T); 6 and 7, *T. rangeli* Triat-1 transfected with pTREXn-GFP5(S65T) grown with G418; 8 and 9, *T. rangeli* Triat-1 transfected with pTREXn-GFP5(S65T) present in hemolymph recovered from *R. prolixus*; 10 and 11, *T. rangeli* Triat-1 transfected with pTREXn-GFP5(S65T) grown without G418; and 12, *Hansenula wingei* Wickerham, 1950, chromosomes.

from LLC-MK2 cells and metacyclic trypomastigotes grown in LIT medium can efficiently and continuously infect tissue cultures in vitro. The fluorescence could be seen in all intracellular developmental forms (amastigote and trypomastigotes), and in free metacyclic trypomastigotes. The GFP did not seem to be toxic to either the parasites or the cultured monkey cells.

Discussion

In the present work, we used the *T. cruzi* RP to drive stable expression of green (GFP) and red (DsRed) fluorescent proteins in different strains (and groups) of *T. cruzi* and *T. rangeli.* Although previous transient gene expression experiments driven by the RP corroborated the universality of group-2 *T. cruzi* sequences (Nunes et al. 1997), the validity of these data for establishing phylogenic relationships has been questioned (Laurent and Swindle 1999). Here, we used stable gene expression driven by a promoter derived from group-2 strain La Cruz (Martinez-Calvillo 1998) and found that our results did not correlate with species or rDNA group; indeed, we found large differences among *T. cruzi* strains (Table 1). For example, the CL Brener (group-1) strain showed poor marker expression, the JMP (group-2) strain and *T. rangeli* yielded intermediate marker expression, and the YBM (group-2) strain showed the highest level of marker expression. We feel that the relatively high expression of the marker in *T. rangeli* emphasized the phylogenetic proximity of the two species, and the robustness of the group-2 RP in this sort of experiment.

Similar results were observed for ubiquitin regulatory sequences in the pBs:CalB1/CUB01 vector, although in contrast to the pTREX-n-based constructs, integration was a rare event. This differential integration of constructs is not easy to explain, because previous reports indicated that RP-driven *T. cruzi* expression vectors rapidly integrated into the ribosomal locus even when transfected as circular plasmids (Martinez-Calvillo et al. 1997, Vazquez and Levin 1999, Lorenzi et al. 2003). In addition, it is unclear how the pTREX-n-constructs integrated into the *T. rangeli* genome, because this did not occur at the ribosomal or GADPH loci. In some strains (Fig. 5, lanes 8 and 9), integration was associated with a molecular weight increase of some bands, which may indicate a multiple tandem repeat insertion of pTREX-n GFP (Lorenzi et al. 2003).

In summary, the pTREX-n vector has several desirable features: efficient transcription and adequate *trans*-splicing lead to high levels of expression, constructs are rapidly and stably integrated, and the vector is fairly universal. These properties allowed us to produce stable *T. cruzi* and *T. rangeli* GFP-tagged cells, which we used to follow the course of infection in *R. prolixus.* In single infections, both parasites completed their expected developmental cycles (D'Alessandro-Bacigalupo and Saravia 1992, Kollien and Schaub 2000) and were easy to visualize fluorescently. In bugs that were fed blood containing 2,000 GFP-*T. rangeli* cells per milliliter, dividing epimastigotes and round forms were concentrated in the insect's slender gut. At day 20 postinfection, a massive crossing of GFP parasites toward the insect hemolymph occurred, and the high degree of pleiomorphism of *T. rangeli* cells in culture was reduced to two forms: elongated epimastigotes and ring-shaped intrahemocyte cells. These results support those of Añez

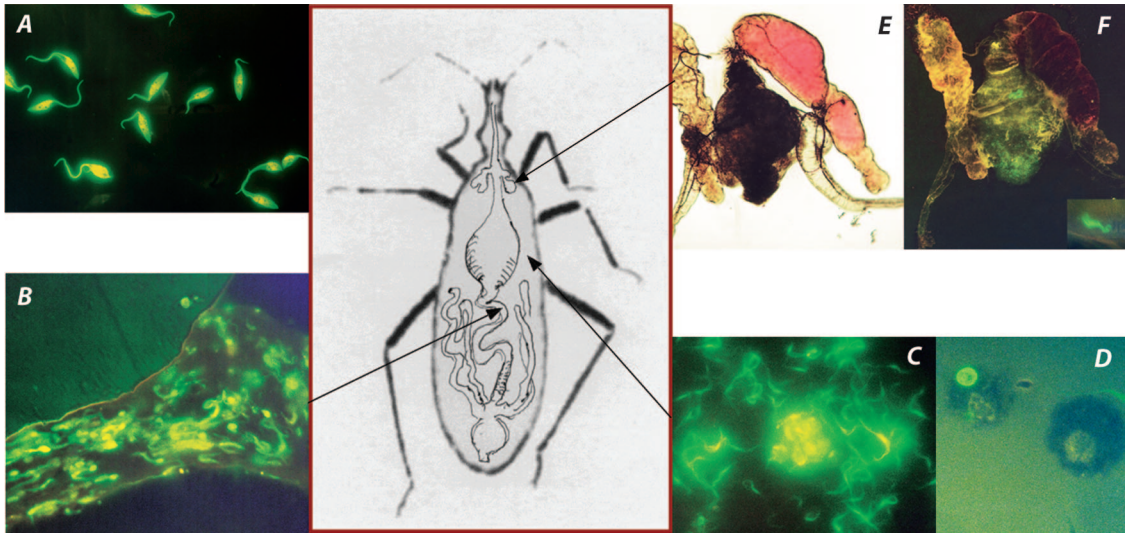

Fig. 6. GFP-tagged *T. rangeli* infections of *R. prolixus.* (A) *T. rangeli*-GFP epimastigotes in culture (used in artificial infection); magnification, 400×. (B) Middle gut 7–19 d postinfection; magnification, 100×. (C and D) Hemolymph extra- and intracellular epimastigotes 19 d postinfection; magnification 100×, 400×. (E and F) Salivary glands (SG) 27 d postinfection; magnification 100×, insert 400×.

(1983) by using nontagged parasites. A week later, the insect salivary glands presented typical infective metacyclic forms and elongated epimastigotes. *T. rangeli* cells were also observed at the insect rectum or in feces, but these were noninfective forms.

Single *T. cruzi* infections reproduced the developmental pattern of this parasite (data not shown). During the mixed infection, parasites retained their developmental patterns. For example, no DsRed-*T. cruzi* cells crossed the insect's gut epithelium, even when large numbers of GFP-*T. rangeli* cells were actively doing so. This evidence is consistent with the existence of specific invasive mechanisms for *T. rangeli*, similar to those described for *Plasmodium* (Ghosh et al. 2001). Also, high numbers of *T. cruzi* and *T. rangeli* cells were observed next to each other, without mixing

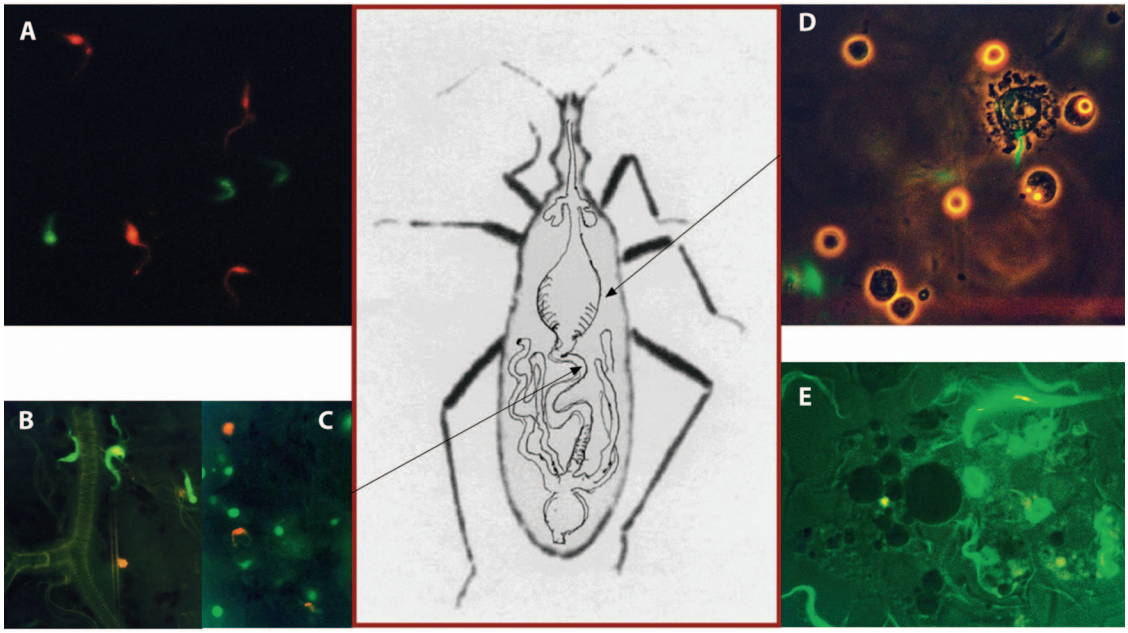

Fig. 7. *T. cruzi*-DsRed/*T. rangeli*-GFP mixed infections of *R. prolixus.* (A) 50:50 mix of *T. cruzi*-DsRed/*T. rangeli*-GFP cultured epimastigotes; magnification, 400×. (B and C) Mid gut mixed infection, 7–20 d; magnification, 400×. (D and E) *T. rangeli* in hemolymph at 21 d postinfection; magnification, 400×.

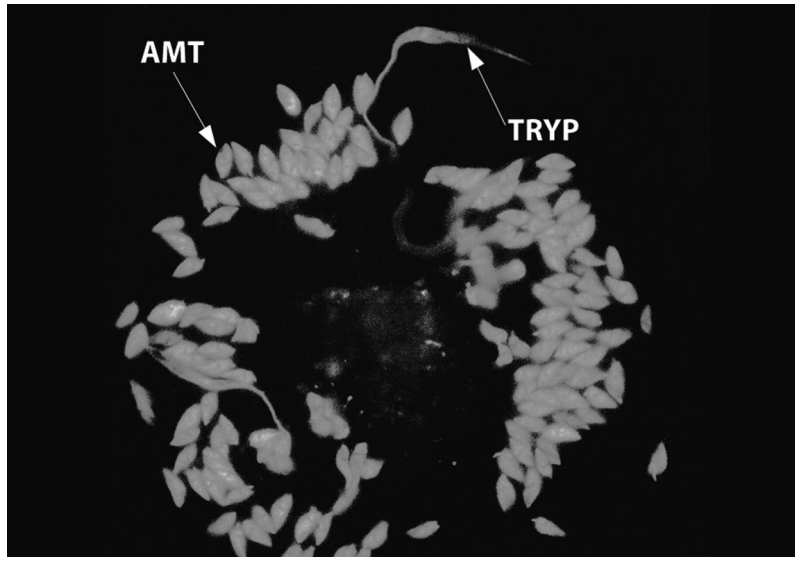

Fig. 8. *T. cruzi* in vitro intracellular cycle. LLCMK2 cells infected with T. cruzi YBM/GFP. Intracellular amastigotes (AMT) differentiating to trypomastigotes in the cytoplasm and intracellular trypomastigotes (TRYP) are observed; magnification, 1000×.

colors or making special contacts. Although this suggests that there is no physical interaction between the two, we cannot exclude chemical interactions and competition for nutrients. In this regard, it would be interesting to investigate whether properties such as infectivity and virulence are affected by coinfection.

Finally, infection of LLC-MK2 cells with GFP-*T. cruzi* YBM strain revealed (Fig. 8) that parasites completed their intracellular cycle without any apparent alteration. Because tagged parasites are the only ones detected under fluorescent microscopy, experimental infections can be done with nonaxenic triatomines captured in the wild. Thus, the ability of a parasite to multiply within a wild-caught bug can be tested, which may help us study vectorial capacity, the epidemiology of emerging triatomine species, and their relationships with parasite strains. Similar observations were previously made for *Leishmania donovani* Ross, 1903 (Guevara et al. 2001). Recently, the conditional expression of GFP in different genetic backgrounds has been applied to the analysis of genetic exchange in *T. brucei* that takes place within the insect vector (Bingle et al. 2001). Overall, tagged parasites can be used to determine cell-to-cell interactions, quantify parasite penetration, discriminate between previous infections and reinfection, and identify the presence of parasitic cells at chronic stages of infections in animal models.

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