RNA (2000), *6*:1069–1076. Cambridge University Press. Printed in the USA.

METHOD

Genetic interference in *Trypanosoma brucei* by heritable and inducible double-stranded RNA

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ABSTRACT

The use of double-stranded RNA (dsRNA) to disrupt gene expression has become a powerful method of achieving RNA interference (RNAi) in a wide variety of organisms. However, in *Trypanosoma brucei* this tool is restricted to transient interference, because the dsRNA is not stably maintained and its effects are diminished and eventually lost during cellular division. Here, we show that genetic interference by dsRNA can be achieved in a heritable and inducible fashion. To show this, we established stable cell lines expressing dsRNA in the form of stem-loop structures under the control of a tetracycline-inducible promoter. Targeting α-tubulin and actin mRNA resulted in potent and specific mRNA degradation as previously observed in transient interference. Surprisingly, 10-fold down regulation of actin mRNA was not fatal to trypanosomes. This type of approach could be applied to study RNAi in other organisms that are difficult to microinject or electroporate. Furthermore, to quickly probe the consequences of RNAi for a given gene we established a highly efficient in vivo T7 RNA polymerase system for expression of dsRNA. Using the α-tubulin test system we obtained greater than 98% transfection efficiency and the RNAi response lasted at least two to three cell generations. These new developments make it possible to initiate the molecular dissection of RNAi both biochemically and genetically.

Keywords: double-stranded RNA; inducible expression; RNA interference; T7 RNA polymerase; trypanosome

INTRODUCTION

RNA interference (RNAi) is a recently described phenomenon whereby gene-specific double-stranded RNA (dsRNA) induces degradation of target mRNA with consequent sequence-specific inhibition of gene expression. Originally reported in *Caenorhabditis elegans* (Fire et al., 1998), RNAi has been described in organisms as diverse as *Trypanosoma brucei* (Ngo et al., 1998), *Planaria* (Sanchez Alvarado & Newmark, 1999), *Hydra* (Lohmann et al., 1999), and *Drosophila* (Misquitta & Paterson, 1999). In plants, a variety of RNA-mediated silencing mechanisms are referred to as posttranscriptional gene silencing (PTGS) (Wassenegger & Pelissier, 1998), whereas PTGS in the fungus *Neurospora* has been termed "quelling" (Cogoni et al., 1996). The mechanism of RNAi is at present poorly understood. Given the gene-specific nature of RNAi, dsRNA needs to interact specifically with its target mRNA. Whether there exist cellular factors that enhance dsRNA–mRNA recognition and how and in which compartment mRNA degradation is initiated are among the open questions. Recently, several genes involved in the RNAi pathway have been identified in *C. elegans*, namely the *rde* (Tabara et al., 1999) genes and *mut-7* (Ketting et al., 1999). *rde-1* is part of a multigene family with homologs in plants, animals, and in fission yeast, but its precise function is not known. The *mut-7* gene bears the signature motif of RNase D and might represent one of the ribonucleases involved in mRNA degradation. Interestingly, some *rde* as well as *mut-7* mutant animals have enhanced levels of transposon mobilization, suggesting that in *C. elegans* one of the functions of RNAi might be to protect the organism from transposition damage by mobile elements. In quelling-deficient *Neurospora* cells, the mutant gene *qde-1* has similarity to RNA-dependent RNA polymerase of plants, suggesting that RNA synthesis by this enzyme might

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contribute to the PTGS response in this organism (Cogoni & Macino, 1999a). Furthermore, recently another gene *qde*-3, a member of the RecQ DNA helicase family, has been shown to be required for the activation and maintenance of gene silencing in *Neurospora crassa* (Cogoni & Macino, 1999b).

To induce RNAi, dsRNA is delivered into cells using various methods depending on the organism's biology: the most common one being microinjection of synthetic dsRNA. In *C. elegans* it is also possible to soak worms in dsRNA solutions (Tabara et al., 1998), and by an unknown mechanism, the dsRNA makes its way into cells. In trypanosomes we have shown that transient expression of tubulin dsRNA from plasmid constructs or electroporation of synthetic tubulin dsRNA causes degradation of tubulin mRNA (Ngo et al., 1998). This resulted in a transient block of tubulin synthesis with the arrest of cytokinesis and consequent accumulation of cells with two nuclei, two kinetoplasts (mitochondrial genome), two basal bodies, and two flagella. After tubulin synthesis resumed, trypanosomes progressed through the cell cycle one more time, duplicated basal bodies and flagella, and carried out nuclear and kinetoplast division. At this point long slender trypanosomes became large almost spherical cells, which we named "FAT" cells. Although the FAT phenotype was terminal, in that trypanosomes never regained the ability to divide, degradation of tubulin mRNA was only transient, whether induced by episomal expression or electroporation of dsRNA. This is because transcription of plasmid DNA ceased a few hours after transfection and we found that the electroporated dsRNA disappeared from cells a few hours after electroporation, most likely because it was degraded. We have further shown that RNAi in trypanosomes is not restricted to tubulin mRNA, but affects a variety of mRNAs, including abundant mRNAs coding for actin or the paraflagellar rod (PFR) protein (Deflorin et al., 1994), and rare mRNAs like those coding for the capping enzyme or the large subunit of RNA polymerase (Pol) II (H. Li and G. Mair, unpubl. observations).

To use RNAi as a method for inhibition of gene function and to study the interference mechanism in *T. brucei*, we needed to devise more efficient and more permanent systems to express dsRNA. Here we report the establishment of inducible and heritable RNAi through the expression of dsRNA in the form of gene-specific stem-loop structures under the control of the tetracycline-inducible PARP promoter. Moreover, we have discovered that electroporation of trypanosomes with plasmids expressing tubulin dsRNA from opposing T7 RNA Pol promoters leads to nearly 100% transfection efficiency. With this system it is possible to obtain homogeneous cell populations expressing dsRNA, thus allowing rapid scoring of dsRNA-induced phenotypes. These newly developed tools will allow the molecular dissection of RNAi in trypanosomes both genetically and biochemically.

RESULTS

Inducible expression of α-tubulin dsRNA

In *T. brucei* regulated gene expression can be achieved using the system originally described by Wirtz and Clayton (1995), and subsequently modified to afford expression of highly toxic products (Wirtz et al., 1999). The PARP promoter was rendered responsive to the tetracycline (Tet) repressor by addition of the Tet operator. The appropriate constructs are transfected into a recipient cell line expressing the Tet repressor and are targeted to the nontranscribed ribosomal DNA spacer, which is a transcriptionally silent region of the genome. To establish a regulated system for dsRNA expression, we constructed two insertion vectors, pLewFAT and pLewFATSH (Fig. 1), both derivatives of pLew79 (Wirtz et al., 1999). The important feature of pLewFAT and pLewFATSH is the presence of two copies of the α-tubulin 5′ UTR, one in the sense and the other in the antisense orientation, relative to the PARP promoter. These sequences are separated by a stuffer fragment of about 700 bp, which is necessary for propagation of the plasmids in bacteria due to instability of adjacent long inverted repeats (we refer to the tubulin sequences plus the stuffer fragment as the FAT DNA). We used the 5′ UTR sequence of α-tubulin mRNA as a proof of principle, as we previously showed that dsRNA homologous to the α-tubulin 5′ UTR efficiently targets mRNA degradation, whether transiently expressed from plasmid constructs or electroporated as synthetic dsRNA (Ngo et al., 1998). Transcription of FAT DNA from the PARP promoter will generate an RNA molecule of about 1,000 nt, in which the α-tubulin sequences form a stem structure of 113 nt interrupted by a single-stranded loop consisting of the stuffer fragment sequence. The difference between pLewFAT and pLewFATSH is the presence in pLewFAT of an expression cassette for the green fluorescent protein (gfp) upstream of the FAT DNA. This is the same arrangement present in our prototypical construct pGFPFAT, which we have shown works well in transient expression assays.

pLewFAT and pLewFATSH were linearized in the rDNA targeting sequence and transfected by electroporation into 29.13.6 procyclic trypanosome cells. Cells were selected for resistance to phleomycin and cloned on agarose plates. Several cell lines derived from insertion of the pLewFAT construct, termed FAT inducible (FATI) cells, were expanded in liquid culture and expression of the FAT DNA induced with Tet. The FAT phenotype became evident in all cell lines tested about 22 h after Tet addition, and by 48 h virtually all cells in the culture were FAT (Fig. 2A, panels a and b). By day 4, large clumps of cells had formed that were visible by simple inspection of the flasks (Fig. 2A, panel d), and eventually the cells died. We analyzed by Northern blotting the amount of α-tubulin mRNA 22 h after in-

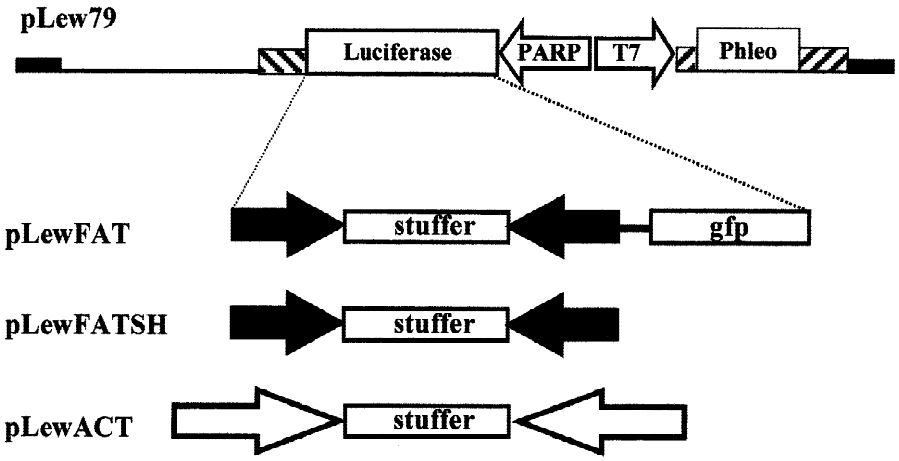

FIGURE 1. Structure of the plasmid constructs for expression of dsRNA. A schematic representation of plasmid vector pLew79 (Wirtz et al., 1999), which was used for dsRNA expression, is shown at the top. This plasmid contains the Tet-regulatable PARP promoter (PARP); the phleomycin resistance gene (Phleo) under the control of a T7 RNA Pol promoter; the nontranscribed rDNA spacer (solid boxes at the ends of the linear vector), which mediates insertion into the rDNA locus; and the fire fly luciferase gene. pLewFAT, pLewFASH, and pLewACT were constructed by substituting the luciferase gene with the appropriate insert as diagramed below pLew79. The insert of pLewFAT was directly isolated from pGFPFAT DNA (Ngo et al., 1998) and consisted of the following elements (in a 5′-to-3′ direction relative to the PARP promoter): the gfp gene followed by signals required for 3′ end processing; the α-tubulin mRNA 5′ UTR in the sense orientation; the stuffer fragment of about 700 nt in length, which is derived from the spliced leader RNA gene repeat; and the α-tubulin mRNA 5′ UTR in the antisense configuration. In the pLewFATSH construct the insert comprises only the sense and antisense α-tubulin 5′ UTR separated by the stuffer fragment. pLewACT is similar in structure to pLewFATSH except that it contains the sense and antisense actin coding sequence from nt 4–426 after the ATG. Solid arrows: α-tubulin mRNA 5′ UTR; open arrows: actin mRNA coding sequences; hatched boxes: sequences containing pre-mRNA processing signals. The drawing is not to scale.

duction with Tet (Fig. 2B). In all cases the FATI cell lines, whether established with the pLewFAT (Fig. 2B) or the pLewFATSH construct (FATSHI cells; data not shown, but see Fig. 3), displayed about a 10-fold decrease in the amount of steady-state α-tubulin mRNA as compared to the uninduced controls. Next, we asked whether the degradation of α-tubulin mRNA could be manipulated by varying the concentration of Tet. Figure 3 shows the results of titrating the amount of Tet in the culture between 0.001 and 10 μg/mL. Degradation of α-tubulin mRNA became evident at 0.5 μg/mL Tet and slightly increased at the higher concentrations tested. The same RNA samples were also hybridized with a stuffer fragment-specific probe to detect transcripts derived from the FAT DNA (Fig. 3). Clear hybridizing bands of about the expected size of 1,000 nt were detected at 0.5 μg/mL Tet and at higher concentrations. Lastly, the cells were scored at the optical microscope for acquisition of the FAT phenotype. FAT cells formed at 0.5 μg/mL Tet and higher concentrations, but very few if any were present at lower concentrations (data not shown). Thus, there appeared to be a threshold Tet concentration below which the FAT phenotype did not appear. This threshold concentration correlated with the detection of the FAT phenotype, degradation of α-tubulin mRNA or the appearance of FAT DNA transcripts.

Inducible expression of actin dsRNA

To determine whether other mRNAs could be targeted using the inducible strategy described above, we chose actin mRNA because in our previous experiments actin mRNA could be efficiently destroyed by electroporating homologous dsRNA, and because we wished to address the possible function of actin in trypanosomes. There are two identical actin genes in *T. brucei* and, although actin mRNA is easily detected, the actin protein and its function have been elusive so far. In the vector pLewACT (Fig. 1) the sense and antisense actin sequences were derived from 426 nt immediately downstream of the actin translation initiation codon. After transfection of pLewACT, clonal cell lines (ACTI cells) were established and the amount of actin mRNA was measured by Northern blot analysis at different times after induction with Tet (Fig. 4). We observed a decrease of about 10- to 20-fold in actin mRNA after 8 h of culturing cells in the presence of Tet and this did not change significantly after longer culturing times. At time points earlier than 8 h, degradation of actin mRNA was not substantial (data not shown). Hybridization to the stuffer fragment revealed transcripts only in the induced samples, but these transcripts had an apparent mobility of about 1,000 nt and thus were shorter than the expected size of about 1,600 nt. Surprisingly, the

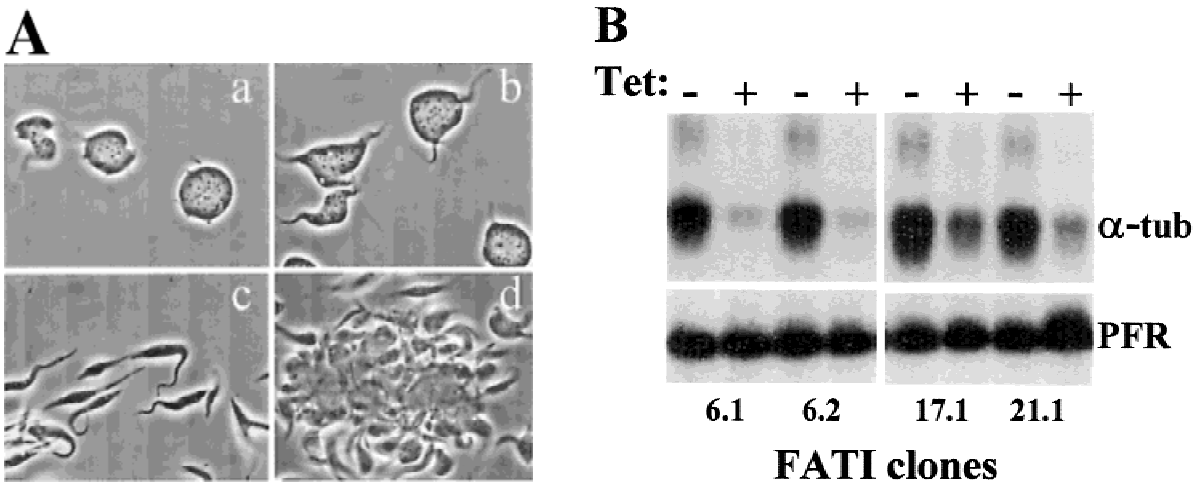

FIGURE 2. **A**: Inducible expression of the FAT phenotype. Trypanosome FATI cells clone 6 were incubated with 1.0 μg/mL Tet for 48 h (panels a and b) or 96 h (panel d) or with an equivalent amount of 70% ethanol (panel c). Living cells were mounted for microscopy and phase images are shown. **B**: Inducible down regulation of α-tubulin mRNA. Individual FATI clones were incubated with (+) or without (−) Tet for 22 h. The identity of each clone is indicated below the lanes. Total RNA was extracted, fractionated through a 1.2% agarose-formaldehyde gel, and processed for Northern blot analysis. α-tubulin mRNA was detected by hybridization with an antisense DNA probe of the entire α-tubulin coding region. The same blot was rehybridized with a paraflagellar rod (PFR) gene probe to control for loading differences.

growing characteristics of ACTI cells were indistinguishable without and after induction with Tet and induced ACTI cells could be maintained in culture for many days without any appreciable loss of viability. We tried without success to detect trypanosome actin in whole-cell lysates and cytoskeletal fractions by western blotting using commercially available monoclonal and polyclonal antibodies, as well as using polyclonal antibodies against *Toxoplasma gondii* actin.

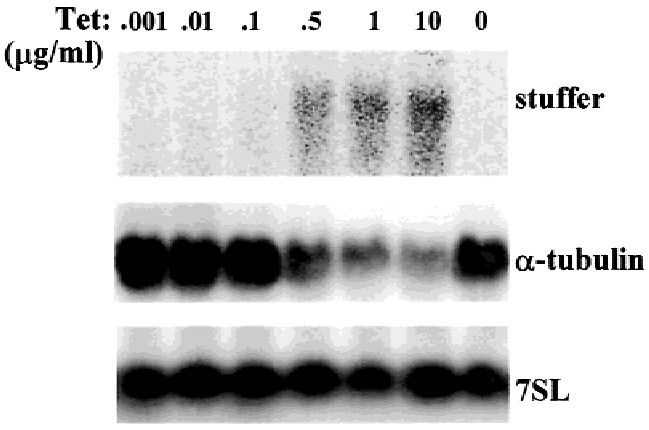

FIGURE 3. Down regulation of α-tubulin mRNA levels and expression of FAT DNA-derived transcripts in the presence of different Tet concentrations. FATSHI clone 8 cells were incubated with the indicated concentrations of Tet and total RNA was prepared after 24 h. The RNA was fractionated as described in Figure 2B and hybridized with an antisense α-tubulin coding region probe (α-tubulin panel), a probe specific for the stuffer DNA fragment (stuffer panel) and an antisense 7SL RNA oligonucleotide (7SL panel).

Efficient T7 RNA polymerase-mediated expression of dsRNA

T7 Pol is a powerful enzyme with an activity far stronger than any of the eukaryotic RNA polymerases. Expression mediated by T7 RNA Pol in trypanosomes had been achieved several years ago and was recently demonstrated to be at least fivefold greater than the one mediated by the PARP promoter, a strong promoter recognized by a Pol I-like enzyme, and about 100-fold greater than expression mediated by Pol II (Wirtz et al., 1994, 1998). Furthermore, Fire and colleagues have shown that dsRNA can be produced in bacteria expressing T7 RNA Pol upon transformation with plasmid pPD129.36 containing two opposing T7 promoters (Timmons & Fire, 1998). We therefore decided to test whether T7 RNA Pol could be used for obtaining efficient expression of dsRNA in trypanosomes.

First, we investigated whether down regulation of α-tubulin mRNA could be induced by independent but simultaneous T7 RNA Pol-mediated expression of sense and antisense sequences. Plasmids p1T7-AT3S and p1T7-AT3A (Fig. 5A) contained a single T7 promoter and encoded the sense and antisense sequence, respectively, of an α-tubulin fragment from position 701 to position 1050 of the coding region. Electroporation of these constructs singly or in combination produced the following results. Transfection of p1T7-AT3A gave rise to about 8% FAT cells, whereas no FAT cells could be detected with p1T7-AT3S. Cotransfection of both plasmids produced about 85% FAT cells, consistent with the requirement of expression of both sense and antisense sequences for induction of RNAi.

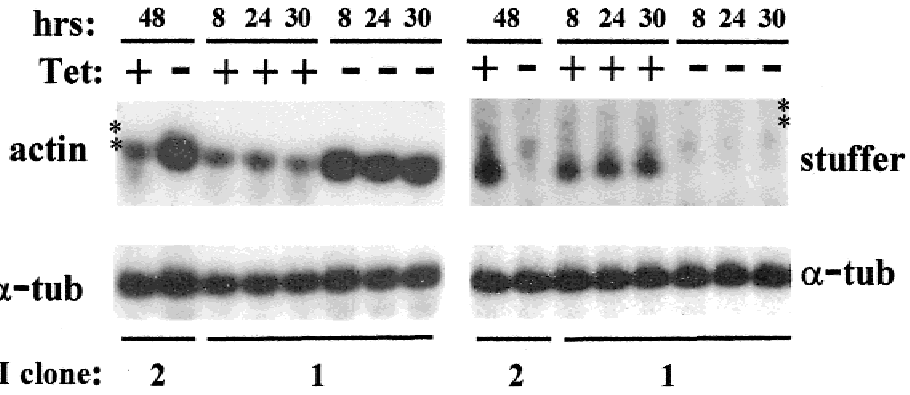

FIGURE 4. Inducible down regulation of actin-mRNA. ACTI cells clones 1 or 2 were incubated in the presence (+) or absence (−) of Tet (1.0 μg/mL). After the indicated periods of time, total RNA was isolated and Northern blot hybridization was performed with an actin antisense DNA probe (actin panel), a stuffer fragment-specific probe (stuffer panel), and a α-tubulin mRNA probe (α-tubulin panel). The positions of the second and third smallest rRNA molecules are indicated by asterisks (1.3 and 1.6 kb).

Next, we inserted the AT3 α-tubulin sequences in the double T7 promoter plasmid pPD129.36 to generate p2T7-AT3 (Fig. 5A). We electroporated different amounts of p2T7-AT3 into 29.13.6 cells that express T7 RNA Pol and the amount of α-tubulin mRNA was measured after 3 h (Fig. 5B). The amount of α-tubulin mRNA decreased with as little as 5 μg of plasmid DNA and reached a minimum between 10 and 25 μg of DNA. After 24 h the cultures were also scored for the presence of FAT cells. There was a very good correlation between the extent of α-tubulin mRNA down regulation and the percentage of FAT cells (Fig. 5B), in that electroporation of 5 μg of plasmid DNA generated about 40% FAT cells, whereas with 25 μg of DNA about 98% of the cells in the culture had turned FAT. Because untransfected cells divide twice during the 16-h incubation required to form FAT cells, the efficiency of transfection was very close to 100%. We next examined the kinetics of transcription of plasmid p2T7-AT3 and the duration of α-tubulin mRNA down regulation by Northern blot analysis (Fig. 6). To detect antisense p2T7-AT3 transcripts we used a probe complementary to plasmid sequences immediately upstream of the T7P1 promoter. As shown in Figure 6A, a heterogeneous collection of RNAs, ranging in size from a few hundred to several thousand nucleotides, appeared as early as 1 h posttransfection, reached a maximum between 1 and 3 h and started to decline thereafter. p2T7-AT3 transcripts could be detected as late as 22 h after transfection, although in reduced amounts. Similar results were obtained for the sense transcripts (data not shown). During the same time course the amount of α-tubulin mRNA began to decline 3 h after electroporation, stayed low for up to 15 h, and only at 22 h did we notice a slight increase (Fig. 6).

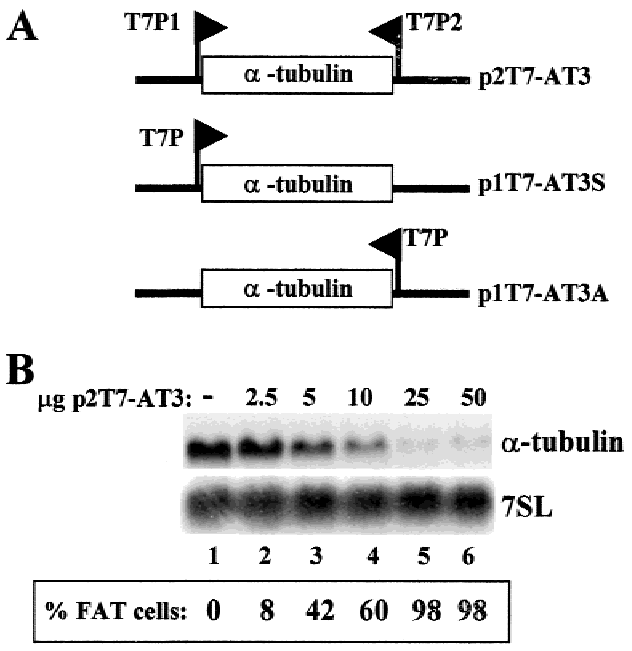

FIGURE 5. Expression of dsRNA mediated by T7 RNA Pol. **A**: Schematic representation of vectors p1T7-AT3S, p1T7-AT3A, and p2T7-AT3. T7P1 and T7P2 indicate the two opposing T7 RNA Pol promoters. α-tubulin indicates the α-tubulin coding region fragment from nt 701–1050 after the ATG. **B**: Efficient degradation of α-tubulin mRNA mediated by T7 RNA Pol expression of dsRNA. The indicated amounts of p2T7-AT3 DNA were electroporated into approximately 10^8 29.13.6 cells, and total RNA was prepared 3 h posttransfection. 5 μg of total RNA were resolved on a 1.2% agarose/formaldehyde gel and processed for Northern blot analysis. The membrane was hybridized with an α-tubulin probe that did not recognize the AT3 tubulin sequence of the transfected DNA (α-tubulin panel). As a control, the blot was rehybridized with an oligonucleotide probe complementary to 7SLRNA (7SL panel). Lane 1: RNA derived from control cells electroporated with 50 μg of pPD129.36 vector DNA.

DISCUSSION

The experiments reported in this article establish two new expression systems for studying RNA interference in trypanosomes, namely an inducible system based on the Tet-regulatable PARP promoter and a powerful

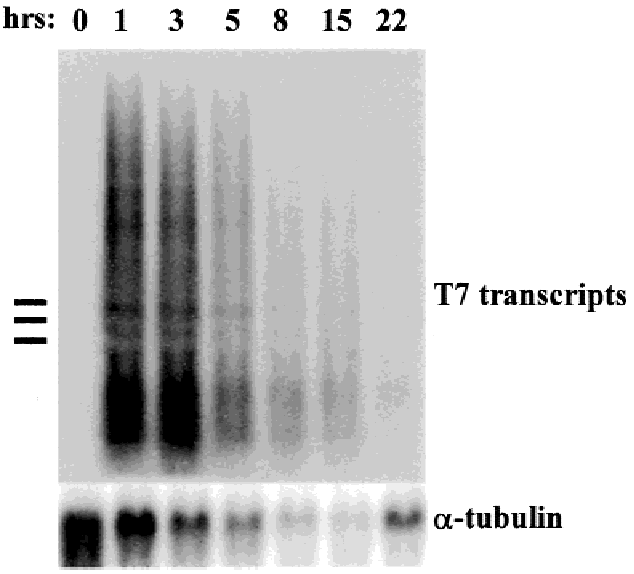

FIGURE 6. Time course of expression of T7 RNA polymerase transcripts and down regulation of α-tubulin mRNA. Twenty micrograms of p2T7-AT3 DNA were electroporated into 10^8 29.13.6 cells and total RNA was prepared at different time points as indicated above each lane. After processing the RNA for Northern blot, duplicate filters were hybridized to either a sense plasmid probe derived from sequences upstream from the T7P1 promoter (panel T7 transcripts) or with an α-tubulin probe as described in Figure 5B (panel α-tubulin). Bars on the side indicate the positions of the three large rRNAs (1.3, 1.6, and 2.1 kb).

transient expression system based on transcription in vivo by T7 RNA Pol.

Inducible RNAi

Using α-tubulin or actin constructs predicted to form dsRNA structures upon transcription from the Tet-PARP promoter, we were able to detect a 10–20-fold down regulation of the amount of α-tubulin or actin mRNA present in steady-state conditions. The magnitude of this effect was comparable to what we observed by transfection of synthetic dsRNAs (Ngo et al., 1998). In the case of FATI cells, we found a threshold Tet concentration required to induce α-tubulin mRNA degradation as well as the FAT phenotype, most likely because RNAi requires robust production of dsRNA. Interestingly, coincident with the activation of RNAi we were able to detect transcripts derived from the stuffer DNA present in our constructs. Although we have not analyzed the structure of these transcripts in detail, it is likely that they are involved in the induction of RNAi. Stuffer fragment-containing transcripts were also present in ACTI cells, although their apparent molecular weight was lower than expected. This might be the result of either aberrant electrophoretic mobility due to the long 450-nt stem structure, which we know from other experiments is very difficult to melt, or to RNA processing events occurring within the actin sequences. Whatever the reason for this phenomenon, it did not affect the extent of degradation of actin mRNA.

It is important to note that in our experiments we always observed a residual amount of α-tubulin or actin mRNA resistant to degradation. This "resistant" mRNA might be located in a cellular compartment where RNAi might not be active, or it might be sequestered from the RNAi degradation machinery. Indeed, we have reported that tubulin polycistronic pre-mRNAs are not subject to degradation by homologous dsRNA (Ngo et al., 1998). Thus, it is possible that a portion of the resistant mRNA represents newly synthesized mRNA that is still located in the nucleus. Alternatively, the resistant mRNA might be an inherent feature of the system, if the rate of synthesis of mRNA is higher than the rate of mRNA degradation induced by dsRNA. Whatever the mechanism, it is evident that RNAi in trypanosomes cannot be used at the present time to generate "null" mutants, but rather to down regulate specific gene functions leading to interesting and informative phenotypes, as we have shown for the tubulin genes.

Surprisingly, down regulation of actin mRNA did not produce any ill effects. Trypanosome actin has been an elusive protein so far, in that attempts to detect the protein with heterologous antibodies have failed except in one reported case (Mortara, 1989), which could not be reproduced in our laboratory. There are several explanations for our inability to detect a phenotype in Tet-induced ACTI cells. First, residual amounts of actin might be present and might suffice to support unimpeded cell growth or another protein might substitute for actin function. Second, actin might be dispensable under our culture conditions, and we could not detect a phenotype because we did not use the appropriate assay for actin function in trypanosomes. For instance, in the protozoa *T. gondii,* actin is part of a microfilament system located underneath the plasma membrane, which is required for invasion of host cells (Dobrowolski & Sibley, 1996). At this point the study of trypanosome actin will require the development of specific antibodies and the construction of strains conditionally expressing actin.

T7 RNA polymerase-mediated expression of dsRNA

We have shown that transfection of tubulin sense and antisense sequences from opposing T7 promoters resulted in almost 100% efficiency in inducing the FAT phenotype. This implied that all the cells acquired the plasmid DNA by electroporation. This is the highest efficiency of DNA transfection ever reported in trypanosomes. As a comparison, transfection of 100 μg of a gfp-expressing plasmid never exceeds 10–20% transfection efficiency in our laboratory. Why is transfection of T7 promoter constructs so efficient? One possibility is that expression of T7 plasmids occurs in the cytoplasm. Indeed, some T7 RNA Pol might be present in the cytoplasm of 29.13.6 cells and we know that RNAi in trypanosomes acts primarily on cyto-

plasmic mRNA. If this were true it would imply that entry by electroporation of plasmid DNA in the cytoplasm is very efficient, whereas entry into the nucleus would be the rate-limiting step for plasmids transcribed by endogenous RNA polymerases.

Interestingly, the Northern blots of Figure 6 showed that the extent of expression of p2T7-AT3 transcripts did not correlate with the extent of α-tubulin mRNA degradation. Whereas T7 Pol-derived transcripts peaked between 1 and 3 h after transfection and declined thereafter, maximal α-tubulin mRNA degradation was observed between 8 and 15 h. At this point the abundance of T7 Pol-derived transcripts was at least 10 to 20-fold lower than at the earlier time points. The lack of correlation between the kinetics of T7 transcripts accumulation and of the degradation of α-tubulin mRNA could be due to a variety of reasons. Most attractive is the possibility that the sense and antisense transcripts produced by T7 RNA Pol need to be processed, for instance annealed and trimmed, and/or associated with some limiting factor present in the cell to become "active." In this context it should be noted that the kinetics of α-tubulin mRNA degradation induced by the double T7 expression system is at least five times slower than what we previously observed by electroporating synthetic preannealed dsRNA into cells. It is possible that the single-stranded tails of the dsRNA produced by T7 Pol transcription are detrimental to the ability of these transcripts to induce RNAi. Indeed, we have observed that synthetic dsRNAs with single-stranded tails seem to be less efficient at inducing RNAi (E. Ullu, unpubl. observations) as compared to dsRNAs with paired ends. Lastly, we should also consider the possibility that excess dsRNA inhibits rather than stimulates the RNAi response.

Notwithstanding the above considerations, using the T7 expression system described here it is possible to obtain homogeneous cell populations that transiently express dsRNA. Down regulation of tubulin mRNA lasted for at least 22 h, encompassing 2–3 cell generations under our culture conditions, making this the system of choice over electroporation of synthetic dsRNA for initial testing of phenotypes associated with down regulation of the gene of interest and for rapid analysis of various aspects of RNAi.

The availability of an inducible system for RNAi and the high efficiency of expression of dsRNA mediated by T7 RNA Pol open the possibility of beginning the molecular dissection of the RNA interference mechanism and the identification of gene products that might be involved in the RNAi pathway.

MATERIALS AND METHODS

Cells and transfections

Procyclic *T. brucei* 29.13.6 cells (Wirtz et al., 1999) were used throughout the experiments. The cells were maintained in Cunningham's medium containing 20% fetal bovine serum. Cells were transfected by electroporation as previously described (Ngo et al., 1998). To establish stable cell lines with the dsRNA-expressing constructs, transformants were selected in medium containing 5 μg/mL phleomycin, 50 μg/mL hygromycin, and 15 μg/mL G418. For cloning purposes cells were spread onto agarose plates as described (Carruthers & Cross, 1992), except that plates were incubated at 28 °C in a 5% CO_2 atmosphere. Individual colonies became visible after about 5 days and were transferred in 100 μL of medium in individual wells of microtiter dishes and expanded in liquid medium. Before addition of Tet, cells were diluted 10 times in fresh medium.

Plasmid constructions and RNA analyses

pLewFAT was constructed by replacing the luciferase gene of plasmid pLew79 (Wirtz et al., 1999) with the *Hin*dIII-*Bgl*II fragment of pGFPFAT (Ngo et al., 1998). For construction of pLewFATSH the region spanning the sense and antisense α-tubulin 5′ UTR sequences plus the intervening stuffer fragment was PCR amplified using as a template pGFPFAT DNA and inserted in place of the luciferase gene of pLew79. To generate the insert of pLewACT, we first constructed the plasmid clone pACT, containing the actin coding region sequences (nt 4–426 from the ATG) in the sense and antisense configuration on either side of the same stuffer fragment present in pGFPFAT. Next, the ACT DNA was inserted in the pLew79 backbone to generate pLewACT. p1T7-AT3A and p1T7-AT3S were constructed by PCR amplification of the α-tubulin coding region between nt 701 and 1050 (the AT3 insert) using oligonucleotides primers that included a T7 Pol promoter sequence in such a way to obtain sense or antisense expression upon transcription with T7 RNA Pol. p2T7-AT3 was constructed by inserting the AT3 sequences between the *Xba*I and *Xho*I restriction sites of plasmid vector pPD129.36 that contains two opposing T7 RNA Pol promoters (Timmons & Fire, 1998).

RNA extraction procedures and Northern analyses were carried out as previously described (Ngo et al., 1998). The stuffer fragment-specific probe was generated by PCR amplification of a DNA fragment from the nontranscribed spacer of the SL RNA gene repeat (nt 210–367 relative to position 1 of the SL RNA gene).

ACKNOWLEDGMENTS

We wish to thank Andrew Fire for providing plasmid pPD129.36, David Sibley for the polyclonal serum against *T. gondii* actin, and Gunnar Mair for helpful discussions and for critical reading of the manuscript. This work was supported by National Institutes of Health grant RO1-AI28798 and by a Burroughs Wellcome Scholar Award in Molecular Parasitology to E.U.

Received February 11, 2000; returned for revision March 2, 2000; revised manuscript received April 21, 2000

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