7

RNA Interference as a Genetic Tool in Trypanosomes

Vivian Bellofatto and Jennifer B. Palenchar

Summary

RNA interference (RNAi) is a cellular mechanism that is often exploited as a technique for quelling the expression of a specific gene. RNAi studies are carried out *in vivo*, making this a powerful means for the study of protein function *in situ*. Several trypanosomatids, including those organisms responsible for human and animal diseases, naturally possess the machinery necessary for RNAi manipulations. This allows for the use of RNAi in unraveling many of the pressing questions regarding the parasite's unique biology. The completion of the *Trypanosoma brucei* genome sequence, coupled with several powerful genetic tools, has resulted in widespread utilization of RNAi in this organism. The key steps for RNAi-based reduction of gene expression, including parasite cell culture, DNA transfection, RNAi expression, and experimental execution, are discussed with a focus on procyclic forms of *Trypanosoma brucei*.

Key Words: RNAi; RNA interference; trypanosomes; parasitic protozoa; eukaryotic gene expression.

1. Introduction

Endogenous RNA interference (RNAi) machineries, which normally regulate gene expression on many levels, can be manipulated at will to alter specific protein levels inside cells. In contrast to more traditional molecular genetic techniques, the timely and laborious process of producing genetic knockouts at the DNA level can be circumvented, thus facilitating a speedy initial study of gene function. Using RNAi, the essential nature of a gene can be assessed and the biological effects of the ablation of a nonessential gene can be deduced.

From: *Methods in Molecular Biology, vol. 442: RNAi: Design and Application*
Edited by: S. Barik © Humana Press, Totowa, NJ

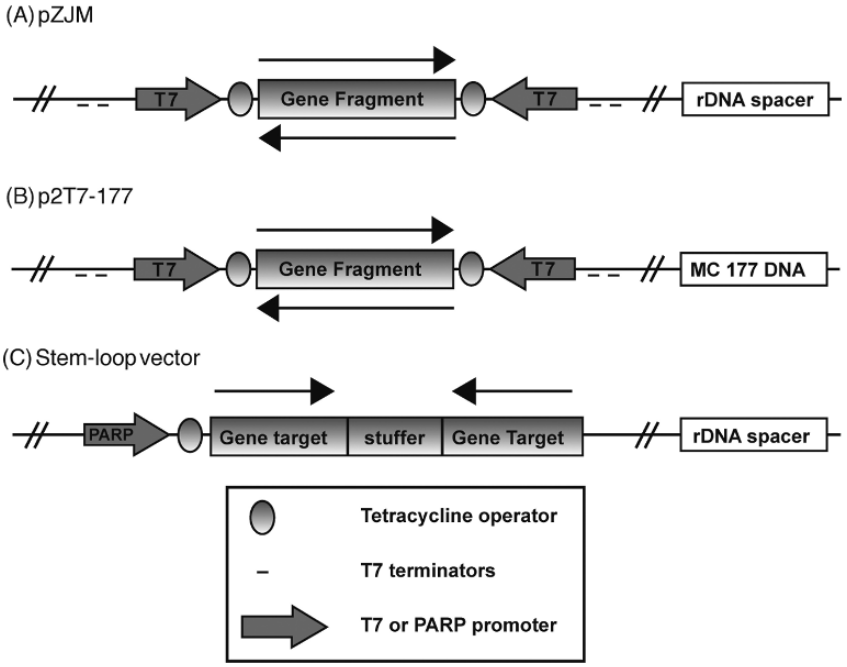

Fig. 1. Important vectors for dsRNA expression in *T. brucei*. (a)–(c). A simple schematic of the vectors used to generate stably transfected cell lines. Both the pZJM ***(23)*** and p2T7-177 ***(17)*** are tetracycline-regulated and contain opposing T7 promoters to generate dsRNA fragments that trigger RNAi. pZJM is targeted to the transcriptionally silent rDNA spacer region of the genome, whereas p2T7-177 is targeted to the minichromosome 177 base-pair repeat region. pZJM has an upstream T7 RNAP-driven phleomycin resistance gene, and p2T7-177 has an upstream rRNA promoter-driven phleomycin-resistance gene; these genes are necessary to maintain the integrated plasmid in the parasite genome. In parts (a) and (b), the short underlines indicate the location of dual T7 terminators ***(15,17)***. In part (c), the stem-loop vector ***(20)*** contains a procyclin (PARP) promoter, is tetracycline-regulated, and is targeted to the rDNA region of the genome as well. The vector is unique in that dsRNA is generated through a stem-loop structure.

The phenomenon of RNAi (*see* **Fig. 1** in **Chapter 4**) was first observed in 1990 when Jorgensen and colleagues attempted to deepen the color of petunias through the introduction and overexpression of a chimeric petunia chalcone synthase gene. Instead, they observed "co-suppression" resulting in white or variegated flowers ***(1)***. Several years later, Mello, Fire and colleagues injected sense and antisense strands of RNA into *C. elegans* ***(2)***. In these experiments, a few double-stranded RNA (dsRNA) molecules per cell were sufficient to silence the homologous gene. This work garnered the pair the 2006 Nobel Prize

in Medicine or Physiology. RNAi has been found in organisms as diverse as humans, flies, fungi, plants, and parasitic protozoa.

The expression of dsRNA triggers the destruction of the complementary mRNA. This dsRNA is a substrate for the ribonuclease Dicer ***(3)***. Dicer cleaves the dsRNA into ~20–25 nucleotide small interfering (si) RNAs. siRNAs enter the RNA induced silencing complex (RISC), where they are unwound. The antisense strand (or guide strand) targets RISC to the complementary mRNA, which is subsequently degraded through RISC nuclease activity.

RNAi in trypanosomes was discovered by the Ullu and Tschudi laboratory ***(4)*** in 1998 during a study designed to address mRNA splicing in the African trypanosome, *Trypanosoma brucei*. In this seminal work, they observed that dsRNA, derived from the sequences that encode the alpha-tubulin genes, caused cells to round up or become "fat." Notably, this was one of the earliest discoveries of RNAi activity in eukaryotic cells, after the initial work on plants and worms ***(2,5)***.

Several trypanosomatids possess endogenous RNAi machinery, including *T. brucei spp*. and *T. congolense* ***(6)***. RNAi is notably absent from *T. cruzi* and *Leishmania spp*. It is unknown if RNAi functions in either in *Leptomonas spp*. or *Crithidia spp*. The combination of available genetic tools, a completed genome sequence, and many pressing trypanosome biology questions make the utilization of RNAi in *T. brucei* highly relevant. The utility of RNAi in this parasite is highlighted by a recent, chromosome-wide analysis of gene function by Subramaniam et al. ***(7)***.

In addition to the importance of *T. brucei* as an infectious agent, this organism as well as the closely related *Leishmania spp*. are excellent model organisms for the study of intracellular processes in eukaryotic cells. *T. brucei* is a single-cell organism that grows rapidly, dividing every ~10 h, axenically in synthetic media. It possesses simplified pathways for many intracellular processes, including having few *cis*-introns in its mRNA and few, if any, discreet promoters for RNA polymerase (RNAP) II-dependent mRNA coding genes. Unlike many other well-studied eukaryotes, it appears to contain few transcription factors, and possibly no transcriptional activators. Equally useful is its overexpression of several molecular traits that are relatively elusive in other organisms. For example, in other eukaryotes, hypermethylated RNA cap structures (designated cap 1 and 2; cap 0 is the me^7GpppG-monomethylated and more common cap structure) are challenging to study due to their low abundance. In *T. brucei*, a hypermethylated cap, designated cap 4, is highly abundant, present on the 5′ end of every mRNA. Indeed, RNAi is being utilized to examine cap 4 biology ***(8)***.

The phenomenon of RNA trans-splicing, the process in which a spliced leader (SL) RNA is added upstream of a translatable region of RNA to produce a stable mRNA, occurs to a different extent in a wide variety of eukaryotes, from

trypanosomes to simple chordates (reviewed in ***9)***. Trypanosomes utilize trans-splicing for the production of every functional mRNA. Most of our knowledge of SL RNA production and function in trans-splicing comes from trypanosome studies. Here, too, RNAi is proving an invaluable tool in understanding SL RNA biogenesis and function ***(10–12)***.

In the *T. brucei* system, adequate genetics are available, including homologous recombination upon DNA transfection, cell lines with small molecule-responsive gene expression (under tetracycline control), and bacteriophage T7 RNAP-dependent RNAi expression, allowing for the facile expression of dsRNA for posttranscriptional downregulation of endogenous mRNAs. Here we describe the current RNAi methodology for *T. brucei*, the ways in which it has been employed, and the important considerations that must be adhered to in data interpretation (*see* **Notes 1–9**).

It is required that the researcher is in a *Trypanosome* laboratory that is already familiar with the growth and the basic genetics of the parasite. Still, in order for the reader to understand the rationale behind the experiments, we will also provide a theoretical background of the major items in **Sections 2** and **3**.

2. Materials

1. Procyclic (tsetse midgut form) trypanosomes, specifically wild-type *T. brucei* strain Lister 427 and its derivatives. This protocol is written for Strain 29-13, which expresses T7 RNA polymerase as well as tetracycline repressor (TetR).

   The specifics of this strain are as follows: The T7 RNAP, a single subunit RNA polymerase, and one copy of the TETR are encoded by the plasmid pLEW13 that is maintained as an integrated molecule in the β-tubulin locus in *T. brucei* by G418 selection. These two genes are maintained by drug selection, as they are coupled to genes that encode resistance to G418 (to maintain the T7 RNAP) and hygromycin (HYG) (to maintain the TETR). The transcriptional abundance of all three foreign genes [T7 RNAP, NEOR (encoding G418R), and TETR] is roughly equal to that of a single β-tubulin transcript. A 3′ untranslated region (3′ UTR) from the *T. brucei* aldolase gene helps destabilize the T7 RNAP mRNA and thus indirectly helps manage the amount of TETR made in the cells. To better control the level of tetracycline-inducible regulation of introduced constructs, the Cross group ***(13)*** added a second TETR gene, under the control of a modified T7 RNAP-dependent promoter, into the "rnp1" locus. The TETR contains a 3′ UTR from actin, which ensures a short half-life to the repressor. This additional DNA is maintained under HYG selection. This HYGR, G418R strain, named 29-13, is to date the optimum strain for RNAi experiments in procyclic *T. brucei*.
2. Parasite growth medium, SDM-79. It is a semidefined medium, supplemented with 10% heat-inactivated fetal calf serum and 7.5 mg/L of hemin ***(14)***.
3. Cytomix: 2 m*M* EGTA (pH 7.6), 120 m*M* KCl, 0.15 m*M* $CaCl_2$, 10 m*M* K_2HPO_4/KH_2PO_4 (pH 7.6), 25 m*M* HEPES (pH 7.6), 5 m*M* $MgCl_2$, 0.5% glucose,

100 µg/mL BSA, and 1 m*M* hypoxanthine. The pH is adjusted to 7.6 (KOH) and sterile-filtered.

4. RIPA buffer: 300 m*M* NaCl, 1% NP-40, 0.25% Na-Deoxycholate, 50 m*M* Tris-HCl (pH 7.4), and 1 m*M* EDTA.
5. Drugs for selection:

Drug	Stock solution	Final concentration in media
Hygromycin	50 mg/mL in PBS	50 µg/mL
G418	50 mg/mL	15 µg /mL
Phleomycin	10 mg/mL	2.5 µg /mL
Tetracycline	1 mg/mL	1 µg/mL

   All stocks are sterile-filtered and stored at –20 °C. Upon thawing, phleomycin is stable for several months at 4 °C, and G418 and hygromycin are stable for up to one year in aqueous solution at this temperature.
6. pZJM vector **(Fig. 1)**: This is the original highly utilized vector for expressing heritable and controllable dsRNA ***(15)***. It utilizes two opposing T7 RNAP promoters under the control of adjacent, *cis*-acting tetracycline operators. An advantage of the pZJM vector is that only a single-step PCR is required to position the dsDNA that encodes the two complementary RNAs between the opposing T7 RNAP promoters. The RNAs anneal inside the nucleus to form a dsRNA that enters the siRNA pathway. Linearized pZJM integrates into one of the rRNA spacer regions in the *T. brucei* genome and is maintained using phleomycin selection. Limiting dilution in selection medium allows for the selection of clonal transfectants.
7. p2T7-177 vector **(Fig. 1)**, a derivative of p2T7 ***(16)***: It was originally designed for integration into minichromosomes, which are generally transcriptionally silent chromosomes in *T. brucei* ***(17)***.
8. Standard cloning protocols and reagents such as restriction enzymes, ligases, phenol/chloroform, LB-agar plates with selective antibiotics, etc.

3. Methods

3.1. T. brucei Cell Culture

1. Store parasites at –80 °C for medium-term storage (a few weeks) and in liquid nitrogen for longer periods. An upright –80 °C freezer is not optimal, as temperature fluctuations decrease cell viability over time.
2. When needed, revive parasites by rapid thawing at room temperature. Add 1 mL of thawed cell stock slowly to 10 mL of prewarmed (27 °C) complete SDM-79 media and incubate at 27 °C for 24 h before challenging with G418 and hygromycin. Grow cells for several days and maintain between 2×10^6 /mL and 1×10^7/mL before using in transfection.

3.2. Choice and Design of dsRNA Sequence

The specific knockdown of a single gene product requires production of dsRNA that is complementary to a unique region of the gene's mRNA. Off-target results

can occur if the dsRNA is close in sequence to nontarget mRNAs. Similarly, if common sequences are used, multiple mRNA can be destroyed simultaneously. For example, using a dsRNA that is complementary to the SL will potentially destroy all *T. brucei* mRNAs. Nonspecific results may be avoided by taking care in designing a dsRNA that only complements the target mRNA. This is best determined by "BLASTing" the dsRNA sequence against the trypanosome genome (http://www.genedb.org). Either a region within the 5′ untranslated region, the open reading frame, or the 3′ untranslated region may be suitable. In all cases, it is optimal to choose a dsRNA sequence that is complementary to ~300–500 contiguous bases of the mRNA.

The RNAit program, found at the Trypanosomatid Functional Analysis Network (http://trypanofan.path.cam.ac.uk/software/RNAit.html), is useful for choosing the unique region of the target gene that will be recognized by the dsRNA. The RNAit program allows the user to circumvent the tedious manual BLAST searching necessary to ensure the suitability of their target sequence. The basic steps are

1. Enter the target sequence.
2. Generate PCR primers based upon melting temperature and length.
3. BLAST the resultant hypothetical PCR product against trypanosome genomic DNA sequence.
4. Parse the results to see whether or not the sequence is suitable for RNAi.

3.3. Construction of RNAi Clones

Although RNAi in trypanosomes was originally observed through the transient transfection of synthetic dsRNA ***(4)***, stable DNA transfection, which allows for heritable expression of dsRNA, the selection of clonal cell lines, and conditional RNAi expression, is more commonly used.

1. Choose from vectors pZJM or p2T7-177 (see their description in **Sections 2** and **4**).
2. Clone the sequence designed in **Section 2.2** in one of the above vectors, grow it as plasmid DNA in *E. coli*, and then linearize by restriction at a single unique site. Linearization facilitates homologous recombination-directed integration into a *T. brucei* chromosome upon DNA transfection.

3.4. Introduction of RNAi Clones

1. Linearize the pZJM and p2T7-177 clones with the restriction enzyme *Not*I. Digest approximately 20 µg of DNA with *Not*I and then purify using a phenol/chloroform extraction step. Ethanol-precipitate the linearized DNA and then resuspend in ~40 µL of sterile deionized water. Determine DNA concentration by absorbance at 260 nm.

2. Grow trypanosomes (*see* **Section 3.1**) to log phase (~6×10^6 cells/mL) and plan to use approximately 1–2 × 10^7 cells per transfection.
3. Harvest the cells from culture by centrifugation at 4 °C, 10 min, at 700 x g, and wash with ZFM ***(22)*** or cytomix at 4 °C.
4. Resuspend cells in ZFM or cytomix (0.5 mL per transfection) and transfer to a prechilled 4-mm electroporation cuvette containing 5–10 μg of DNA that has been linearized and purified (in step 1 above). Immediately subject parasites to electroporation using a BTX600 model electroporator set to a charge of 1.6 kV, 2.5 kV/resistance, and R2 resistance, one pulse. The time constant value after the pulse will be ~0.35. (Using a Bio-Rad Gene Pulser, the settings are 1500V, 25 μF, and 2 pulses.) As a negative control, a transfection containing no DNA is also performed.
5. Immediately transfer cells to 10 mL of prewarmed (27 °C) culture medium and incubate for ~16 h before drug selection is initiated in the next step.
6. For selection, add G418, hygromycin, and phleomycin (for procyclic *T. brucei* strain 29-13 transfected with a phleomycin-resistance dsRNA-synthesizing vector, such as pZJM). The selection process takes approximately one week, with a noticeable difference between the control (no DNA) and the transformed cells at this point.
7. Isolation of clonal cell lines: This is commonly done through limited dilution that may be achieved in several ways (http//tryps.rockefeller.edu). During routine cell culture, SDM-79 media contains 10% fetal calf serum. This amount is increased to 15% during generation of clonal cell lines to increase cloning efficiency. In one approach, cells are diluted to 5 cells/mL and 100-μL aliquots are distributed into a 96-well plate and incubated at 27 °C with CO_2. In ~2 weeks, a noticeable increase in cell density occurs in many of the wells. In a second approach, wild-type Lister 427 parasites are added as "feeder" cells (~0.5–1 × 10^3 feeder cells/mL). Four clonal cell lines should be isolated and used in each RNAi induction experiment.

   In the dilution procedure, note that the cells can be successfully diluted when the culture reaches a density of ~4×10^6 cells/mL. Dilute newly transfected cells relatively slowly (1:1), always into prewarmed media, and eventually transfer to a 25-cm^2 T-flask when the volume reaches ~3 mL. Maintain the clonal cell lines at 3–6 × 10^6 cells/mL. Overdilution of the trypanosomes results in reduced growth or cell death.
8. Storage of clonal cells: Upon production of a stably transfected cell line, the parasites should be stored long-term in liquid nitrogen as follows. Grow parasites to a density of ~8×10^6 cells/mL, then pellet at 700 x g, 10 min, 4 °C. Remove the supernatant immediately and resuspend the parasite pellet in complete SDM-79 medium containing 10% glycerol (no drugs). Each 10 mL of starting culture is resuspended in 1 mL of the glycerol-containing medium. These cells are slowly frozen at –80 °C for three days and then stored in liquid nitrogen.

To use the frozen cells, thaw as described in **Section 3.1.** Selection drugs can be added 16–24 h after thawing.

3.5. dsRNA Expression to Knock Down a Specific Protein

Induction of RNAi is accomplished using a regulated tetracycline-controlled promoter. Tetracycline releases the TETR from the operator region on the vector DNA, thus allowing dsRNA production. As stated above, four clonal cell lines should be used for each induction.

1. On day 0, split the starting culture that is in log phase into two T-flasks. Add tetracycline (or doxycycline) to a final concentration of 1 μg/mL to only one of them, the second flask serving as a noninduced control.
2. Count parasites daily at approximately the same time each day by placing 10 μL of culture onto a Neubauer hemocytometer. At least 100 parasites are counted to obtain meaningful values. Only live cells should be counted; Trypan Blue may be used to differentiate between live and dead cells when counting. In addition, it is crucial to observe cell morphology, as this may result in the development of interesting hypotheses regarding trypanosome biology. Alternatively, cell densities can be determined using a Coulter counter model Z1 (Coulter Electronics). The total number of live cells per mL is calculated and plotted as a function of time since tetracycline induction. Sample data from our laboratories are shown in **Fig. 2**. From the beginning of the experiment, both the RNAi-induced and control cells are monitored by microscopy for cell motility and morphology. When either the induced or noninduced culture density reaches ~8×10^6 cells/mL, it should be diluted to 2×10^6 cells/mL using a prewarmed (27 °C) medium containing the selection drugs (G418, hygromycin, and phleomycin) and tetracycline. This is best done by pelleting the culture and resuspending cells in fresh medium containing freshly added drugs. The length of time required to observe a growth phenotype of an essential gene is variable depending upon target protein stability and protein

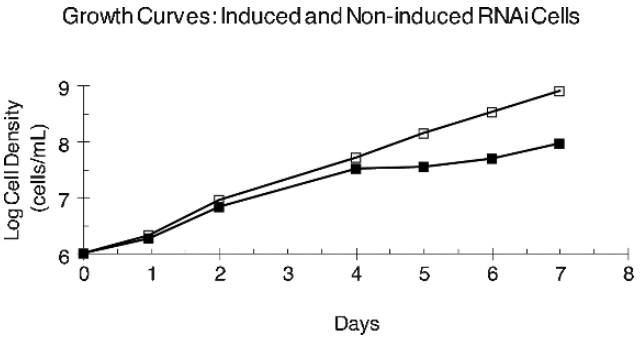

Fig. 2. Growth curve of a procyclic cell line stably transfected with an inducible RNAi construct that ablates an essential gene. At day 0, tetracycline was added to initiate dsRNA production. By day 4, growth of the RNAi-induced cell culture deviates from that of the noninduced control.

concentration. Growth deviations from the noninduced control are generally seen within 1 to 4 days.

3. Follow the induced and noninduced paired cultures daily for 12 days, diluting as necessary to maintain cell densities between 2–8 × 10^6 cells/mL. In some cases, cells harboring an RNAi construct that knocks down an essential protein will begin to recover and resume logarithmic growth rates around day 10. This observation is likely the result of an epigenetic change in genotype, resulting in a loss of RNA interference of the target mRNA. Thus, a gene product is considered essential to cell viability if cells halt log-phase growth within five days of RNAi induction. Clearly, analysis of a gene that produces an essential, albeit long-lived and highly abundant, protein may show a phenotypic effect as a result of protein dilution in the population; therefore, growth arrest may be delayed.
4. To monitor protein levels throughout the experiment, remove approximately 5 × 10^6 cells daily from both induced and noninduced cultures (the volume necessary to achieve this number of cells can be determined from the daily cell count). Centrifuge cells at 1400 x g, 10 min, 4 °C, and discard the supernatant. Resuspend the pellet in 5 mL of chilled (4 °C) PBS and recentrifuge. Finally, resuspend the pellet in 50 μL of RIPA buffer containing the protease inhibitors PMSF, pepstatin, and leupeptin. Vortex briefly and incubate on ice for 10 min, and centrifuge again at 14,000 x g, 10 min, 4 °C. Use the supernatant (containing cell extract from 1 × 10^5 cells/μL) for Western blot analysis. As a general rule, to accurately monitor and compare protein levels between samples, it is necessary that the extract from the same number of cells be loaded into each well of the SDS-PAGE. Using 20 μL of extract for the Western blot analysis is sufficient to visualize protein levels using specific antibodies and the ECL system of detection. Positive controls and analysis of mRNA are described later (*see* **Section 4**).

4. Notes

1. Potential artifacts: The experimental manipulations of DNA transfection, plasmid DNA integration, drug challenge, and dsRNA expression are stressful for the parasites and many elicit biological responses that are artifactual. Thus, it is crucial that interesting results obtained with RNAi assays be followed up with additional genetic and biochemical experiments.

   Additionally, off-target effects must also be considered in all RNAi experiments. The ablation of nontarget mRNA may cause phenotypic changes that are unrelated to the gene of interest.
2. Target stability: RNAi knockdown of target mRNA and protein levels is a function of mRNA synthesis, translation, and turnover rates. Knockdown of a rapidly transcribed and long-lived mRNA is therefore more difficult than the destruction of a low-level and unstable mRNA.
3. Reversion: Researchers often analyze RNAi-induced cells for the reversion of the observed phenotype. Tetracycline is removed, leading to the shutoff of dsRNA, allowing the return of target mRNA and encoded protein. For example, this

experimental procedure has been used to follow the reacquisition of protein in protein localization studies ***(19)***. It is important to ensure that the regrowth of the transfected cell line is not due to the emergence of revertant cells; this is controlled for by the readdition of tetracycline and observation of the original ablation phenotype.

4. Multiple clones: It has been observed that in some cases only one of several clonal cell lines that are maintained in drug selection (i.e., phleomycin for pZJM and p2T7-177 integrants) actually shows ablation of the target mRNA and encoded protein. Thus, it is important to analyze several clones for both mRNA and protein levels to maximize the chance of recovering a desired cell line.
5. Positive controls: A useful positive control is the knockdown of alpha-tubulin, as described initially by the Ullu and Tschudi laboratory ***(20)***. Either the pLEWFAT construct, which contains a dsRNA that complements the 5'UTR of the alpha-tubulin mRNA, or a derivative of the pZJM that produces a dsRNA that is complementary to 650 bp of the coding region of alpha-tubulin, has been used ***(20,23)***. For this control, the 650-bp region is cloned into the vector backbone that is being used for the experiments. A stable cell line containing the alpha-tubulin dsRNA should be generated in parallel with the gene under study. Prior to cloning out the experimental cell lines, the nonclonal tubulin targeted parasites are induced with tetracycline to decrease tubulin protein levels. Cells should appear "fat" by microscopy after 24–48 h of culture. This observation confirms that the transfection and induction steps of the experiment were successful.
6. Other assays: Analysis of specific mRNA and/or protein shutoff is performed using Northern blotting, to assess mRNA levels, or Western blotting, to assess protein levels. For RNA detection, use total RNA, which is ~10% mRNA, following a standard Northern protocol and probing with ^{32}P-labeled DNA used for the transfection step. It is possible to detect both the loss of the target mRNA and the presence of the dsRNA after RNAi induction. It is preferable to assess protein levels, assuming a specific antibody is available, as discussed above.
7. Background expression: Inserting the T7 RNAP-driven, tetracycline-inducible dsRNA-producing vector into this locus results in less "background"-level expression of the dsRNA. Low-level "background" expression can be harmful to the experimental outcome, as it can result in partial inhibition of an essential gene and thus uncontrolled genetic changes during transfected cell selection. To obviate this problem, a more highly controlled ectopic dsRNA, such as that obtainable by cloning into transcriptionally silent minichromosomes, is preferable. In addition, currently available derivatives of the p2T7 vector allow for easy cloning of PCR products (the p2T7^{TAblue} plasmid) ***(18)***. Several vectors are now available for the construction of stably transfected cell lines in which the expression of dsRNA is under the control of tetracycline.
8. Vectors for stem-loop or hairpin dsRNA production: These constructs were used in the early days of trypanosome RNAi experimentation ***(19,20)***. Although constructing the hairpin vectors requires two cloning steps, expression of the hairpin RNA from these vectors is tightly regulated and highly expressed upon

induction ***(21)***. These vectors produce a single RNA molecule that folds back on itself to form a stem-loop structure that then enters the siRNA pathway.

9. RNAi protocols for analysis of gene expression in bloodstream forms of *T. brucei* can be found at http://tryps.rockefeller.edu/ and at http://trypanofan.path.cam.ac.uk.

Acknowledgments

We thank Chris Utter for critical reading of the manuscript. This work was supported by grants AI29478 and AI53835 to V. B., who is also a recipient of a Burroughs Wellcome Fund New Investigator Award in Molecular Parasitology. J. B. P. is supported by the Department of Chemistry at Villanova University.

References

1. Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible co-suppression of homologous genes in trans. *Plant Cell* **2,** 279–289.
2. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391,** 806–811.
3. Hutvagner, G., and Zamore, P. D. (2002). RNAi: Nature abhors a double-strand. *Curr. Opin. Genet. Dev*. **12,** 225–232.
4. Ngo, H., Tschudi, C., Gull, K., and Ullu, E. (1998). Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. *Proc. Natl. Acad. Sci. USA* **95,** 14687–14692.
5. Metzlaff, M., O'Dell, M., Cluster, P. D., and Flavell, R. B. (1997). RNA-mediated RNA degradation and chalcone synthase A silencing in petunia. *Cell* **88,** 845–854.
6. Inoue, N., Otsu, K., Ferraro, D. M., and Donelson, J. E. (2002). Tetracycline-regulated RNA interference in *Trypanosoma congolense*. *Mol. Biochem. Parasitol*. **120,** 309–313.
7. Subramaniam, C., Veazey, P., Redmond, S., et al. (2006). Chromosome-wide analysis of gene function by RNA interference in the African trypanosome. *Eukaryot. Cell* **5,** 1539–1549.
8. Li, H., and Tschudi, C. (2005). Novel and essential subunits in the 300-kilodalton nuclear cap binding complex of *Trypanosoma brucei*. *Mol. Cell. Biol*. **25,** 2216–2226.
9. Palenchar, J. B., and Bellofatto, V. (2006). Gene transcription in trypanosomes. *Mol. Biochem. Parasitol*. **146,** 135–141.
10. Biton, M., Mandelboim, M., Arvatz, G., and Michaeli, S. (2006). RNAi interference of XPO1 and Sm genes and their effect on the spliced leader RNA in *Trypanosoma brucei*. *Mol. Biochem. Parasitol*. **150,** 132–143.

11. Palenchar, J. B., Liu, W., Palenchar, P. M., and Bellofatto, V. (2006). A divergent transcription factor TFIIB in trypanosomes is required for RNA polymerase II-dependent spliced leader RNA transcription and cell viability. *Eukaryot. Cell* **5,** 293–300.
12. Zeiner, G. M., Foldynova, S., Sturm, N. R., Lukes, J., and Campbell, D. A. (2004). SmD1 is required for spliced leader RNA biogenesis. *Eukaryot. Cell* **3,** 241–244.
13. Wirtz, E., Leal, S., Ochatt, C., and Cross, G. A. (1999). A tightly regulated inducible expression system for conditional gene knock-outs and dominant-negative genetics in *Trypanosoma brucei*. *Mol. Biochem. Parasitol*. **99,** 89–101.
14. Cross, G. A., and Manning, J. C. (1973). Cultivation of *Trypanosoma brucei spp.* in semi-defined and defined media. *Parasitology* **67,** 315–331.
15. Wang, Z., and Englund, P. T. (2001). RNA interference of a trypanosome topoisomerase II causes progressive loss of mitochondrial DNA. *EMBO J*. **20,** 4674–4683.
16. LaCount, D. J., Bruse, S., Hill, K. L., and Donelson, J. E. (2000). Double-stranded RNA interference in *Trypanosoma brucei* using head-to-head promoters. *Mol. Biochem. Parasitol*. **111,** 67–76.
17. Wickstead, B., Ersfeld, K., and Gull, K. (2002). Targeting of a tetracycline-inducible expression system to the transcriptionally silent minichromosomes of *Trypanosoma brucei*. *Mol. Biochem. Parasitol*. **125,** 211–216.
18. Alibu, V. P., Storm, L., Haile, S., Clayton, C., and Horn, D. (2005). A doubly inducible system for RNA interference and rapid RNAi plasmid construction in *Trypanosoma brucei*. *Mol. Biochem. Parasitol*. **139,** 75–82.
19. Bastin, P., Ellis, K., Kohl, L., and Gull, K. (2000). Flagellum ontogeny in trypanosomes studied via an inherited and regulated RNA interference system. *J. Cell Sci*. **113,** 3321–3328.
20. Shi, H., Djikeng, A., Mark, T., Wirtz, E., Tschudi, C., and Ullu, E. (2000). Genetic interference in *Trypanosoma brucei* by heritable and inducible double-stranded RNA. *RNA* **6,** 1069–1076.
21. Djikeng, A., Shen, S., Tschudi, C., and Ullu, E. (2004). Analysis of gene function in *Trypanosoma brucei* using RNA interference. *Meth. Mol. Biol*. **265,** 73–83.
22. Bellofatto, V., and Cross, G. A. M. (1989). Expression of a bacterial gene in a trypanosomatid protozoan. *Science* **244,** 1167–1169.
23. Wang, Z., Morris, J. C., Drew, M. E., and Englund, P. T. (2000). Inhibition of *Trypanosoma brucei* gene expression by RNA interference using an integratable vector with opposing T7 promoters. *J. Biol. Chem*. **275,** 40174–40179.