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A doubly inducible system for RNA interference and rapid RNAi plasmid construction in *Trypanosoma brucei*

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Abstract

The most rapid method for the generation of conditional mutants in *Trypanosoma brucei* is the use of RNA interference. A single copy of the target sequence is cloned between two opposing T7 promoters bearing *tet* operators, and the resulting plasmid is integrated into the genome of cells expressing both the *tet* repressor and T7 RNA polymerase. Upon addition of tetracycline, double-stranded RNA is synthesised from the two T7 promoters. Unfortunately, repression of T7 promoter activity may sometimes be insufficient to prevent expression of toxic amounts of double-stranded RNA. We describe here cell lines in which the expression of T7 polymerase is under tetracycline control, and show that regulation of polymerase expression can modulate transcription from a constitutive T7 promoter. In addition we describe a construct containing two copies of the *tn*10 Tet repressor for easy creation of repressor-expressing trypanosomes, and an RNA interference vector which allows "TA" cloning of unmodified PCR products and blue/white selection.

Keywords: *Trypanosoma brucei*; T7 polymerase; RNA interference vector; Tet repressor

1. Introduction

The study of the functions of essential genes in *Trypanosoma brucei* has been greatly facilitated by the use of inducible gene expression. The system relies on trypanosomes that stably express the Tn10 *Tet* repressor [1]. Plasmids containing the gene or RNA that is to be inducibly expressed are designed to integrate into a region of the genome that is usually transcriptionally silent, such as the ribosomal RNA spacer. The plasmids contain one promoter which drives expression of a selectable marker, and a second, inducible promoter bearing two copies of the *tet* operator very close to the transcriptional start site [2,3]. The inducible promoter that has been used most so far is an *EP*1 promoter (from the *EP* procyclin locus).

Down-regulation of the expression of essential genes in *T. brucei* is most easily achieved via RNA interference. A double-stranded RNA containing the sequences of interest is inducibly expressed, and causes destruction of the homologous mRNA [4–8]. Double-stranded RNAs can be obtained in two ways. One option is to insert, downstream of the inducible *EP* promoter, two copies of the specific DNA sequence in opposite orientations, separated by a "stuffer" fragment which enables the plasmid to replicate stably in *E. coli* [4]. Transcription of the (target)-stuffer-(reverse-target) construct generates a stem-loop RNA, and the stem mediates RNA interference. Promoter regulation is relatively tight but several cloning steps are required and some sequence com-

Abbreviations: 3′-UTR, 3′-untranslated region; TETR, Tetracycline-sensitive repressor from transposon Tn10; VSG, Variant surface glycoprotein; EP1, EP1 procyclin; ΔALD, deleted version of aldolase 3′-untranslated region; *CAT*, chloramphenicol acetyltransferase; LUC, firefly luciferase; T7POL, T7 polymerase; TUB, tubulin

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binations resist cloning. Alternatively, the double-stranded RNAs can be synthesised from a single template which is flanked by opposing T7 promoters [5,6]. The latter approach has the advantage of requiring only one cloning step, but "background" expression from the "repressed" T7 promoter is usually higher than for the *EP* promoter [9]. In the worst case, the background dsRNA expression may be so deleterious as to prevent selection of suitable transformants.

We here describe attempts to improve all aspects of RNAi in trypanosomes: creation of repressor-expressing lines, the cloning of PCR products, and the selection of trypanosomes exhibiting tightly-regulated RNAi.

2. Methods

2.1. Plasmid construction

To test 3′-untranslated regions, the plasmid pHD 1034 [10] was used. This contains a chloramphenicol acetyltransferase (*CAT*) cassette linked to a puromycin resistance cassette (*PURO*); transcription is from the ribosomal RNA (*RRNA*) promoter. In pHD 1034 the *CAT* cassette has an actin (*ACT*) 3′-UTR flanked by *Bam* HI and *Sal* I sites. The new 3′-UTRs were amplified from cloned cDNAs [11] using specific oligonucleotides and cloned as *Bam* HI–*Sal* I fragments into similarly cut pHD1034, except for the S83′-UTR which was cloned as a blunted *Bam* HI–*Kpn* I fragment. The boundary sequences (with restriction sites, underlined) for the different 3′-UTRs were as follows: Ribosomal protein S18 (pHD 1201)–GGATCCGAAAGGCTGCCAAGAAAC. . . .. TCTTTCTTTTTACTGAAAGTCGAC; Ribosomal protein L30 (pHD 1202)–-GGATCCTAGGTGTATGCCGTCATG. . .. ATTCAAACCAGCTTAAAAGTCGAC; Ribosomal protein L28 (pHD 1203)–GGATCCAGACAGTCCGCCCAAAGC. . .. CTTTTCGTTTACATTGAGGTCGAC; Ribosomal protein S8 (pHD 1204, blunt-end cloning)–GAGTGGAGAGCCGACCTG. . .. AAACACTACGGTATCGAC; Ribosomal protein S18 (pHD 1205)–GGATCCGCGTGCTCCTCGCAGTGT. . .. CCACCCTGTTGACAGTTTGTCGAC.

The plasmids were transfected into bloodstream trypanosomes and at least 3 clones selected for *CAT* activity measurement.

RNA interference plasmids directed against tubulin, PEX2 and histone H3 were as follows. The PEX2 and XRNB primers were as specified by the Trypanofan programme [12]: PEX2: CTAggatccGCTGTATCCGTTTCGTGGAT and ACCctcgagCAACGCAAGCATCTAAACGA; XRNB: AGGAagatctgcatgcTGCCAAAGTTCGCCTCTTGG and GTgaattcgtcgacCATAATTCAATCATCTGTGCTCC. The Histone H3 primers were TCGAGGACCAAGGAAACCG and ATGCACGTTCACCGCGTAG (the 397 bp target encompasses almost the entire H3 gene) and the tubulin primers were CC*CTCGAG*CCACACCTTCATTGGCAACAACA and CG*GGATCC*CCACCTCTGCGATGGCCG (the 2027 bp target encompasses parts of the α- and β-tubulin genes).

The construction of the vector plasmids used in the remainder of the study are described in the legends to Figs. 1, 2 and 4. Reconstructed sequences are available from the authors and the sequence of p2T7^{Ti}:TAblue is on the trypanofan Web site (http://www.trypanofan.org/).

2.2. Trypanosome culture

Culture and transfection of *T. brucei* lines 427, 927 was as described [11]. In all transfections 10 μg *Not* I-linearised DNA were transfected into 10^7 cells. Selection was initiated the following day and at the same time cells were cloned by limiting dilution. Selecting drug concentrations for bloodstream forms were: hygromycin 5 μg ml^{-1}, G4182 μg ml^{-1}, phleomycin 20 μg ml^{-1} (0.2 μg ml^{-1} for maintenance), puromycin 0.2 μg ml^{-1}. Drug concentrations for procyclic forms were: hygromycin 50 μg ml^{-1}, G41815 μg ml^{-1}, phleomycin 5 μg ml^{-1}, puromycin 2 μg ml^{-1}. Tetracycline was added at 100 ng ml^{-1}.

To test growth of clones, bloodstream trypanosomes were diluted to 10^4 to 10^5 ml^{-1} every 2 days, and procyclics were diluted to 10^5 to 5×10^5 ml^{-1}. Clones were allowed to grow and the number relative to the control counted before the next dilution.

2.3. Westerns, CAT and luciferase assays

Western blots were developed with antibody to the *tet* repressor (obtained from Prof. Dominique Soldati, then at the ZMBH, as a supernatant from a previous blot) and to T7 polymerase (rabbit anti-T7 RNA polymerase antibody affinity purified and used at 1:1000, obtained from Dr. Achim Schnaufer, Seattle Biomedical Research Institute). The control was a cytosolic marker described in [13]. Detection was with ECL (Amersham, Braunschweig) as described by the manufacturer.

CAT and luciferase assays were as previously described [14]. For *CAT* assays, 2×10^6 cells were freeze-thawed in 250 μl assay buffer and 2–20 μl were assayed. Slightly different protein concentrations were adjusted using Bradford Assay. Negative controls of trypanosome homogenate (no *CAT* gene) and buffer were used for background subtraction.

3. Results

3.1. A search for 3′-untranslated regions giving high constitutive expression

Anecdotal reports from various sources suggest that the initial plasmids designed to express the tetracycline repressor, pHD 360 [1] and pHD 449 [2], do not always yield sufficient repressor when transfected into new trypanosome lines. (The lines that are in routine use in our and other laboratories fortuitously integrated two copies