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Regulated processive transcription of chromatin by T7 RNA polymerase in *Trypanosoma brucei*

Elizabeth Wirtz, Maarten Hoek and George A. M. Cross*

Laboratory of Molecular Parasitology, The Rockefeller University, 1230 York Avenue, New York, NY 10021-6399, USA

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ABSTRACT

Inability of T7 RNA polymerase to processively transcribe higher eukaryotic chromatin is interpreted as a correlate of its reported inhibition by nucleosomes on reconstituted templates *in vitro*. We used chromosomally integrated reporter cassettes to examine features of T7 transcription in a lower eukaryotic system. Luciferase reporters were targeted to rDNA in transgenic *Trypanosoma brucei* stably expressing the phage polymerase. Because trypanosome mRNAs are capped by RNA splicing *in trans*, T7 transcription could be gauged by luciferase activity. In contrast to findings from higher eukaryotes, T7 transcription is vigorous and processive on chromatin templates in *T.brucei*, surpassing levels achieved with endogenous promoters, including those recruiting RNA polymerase I. This may be a reflection of intrinsic differences in chromatin structure between differently evolved eukaryotes or of an integration site that is exceptionally permissive for T7 transcription due to a local accessible chromatin conformation. T7 transcription could be manipulated to achieve different levels of constitutive expression, through the use of promoter mutations. Moreover, T7 initiation could be regulated by the prokaryotic Tet repressor and elongation halted by T7 terminator sequences. We have exploited these features to construct a robust inducible expression system, whose utility potentially extends to other *trans*-splicing organisms.

INTRODUCTION

Bacteriophage T7 RNA polymerase (T7 RNAP) is a highly processive single subunit enzyme which has been exhaustively characterized with respect to initiation and termination requirements (1–3). This knowledge has led to numerous attempts to import T7 transcription into heterologous systems as the basis for protein expression. Strategies to establish T7-mediated expression technologies in higher eukaryotic systems, however, have met with limited success for two apparent reasons. First, transcription of higher eukaryotic chromatin by the phage polymerase does not appear to be processive, either *in vivo*, as revealed by the use of transgenic *Drosophila* (4), or when added to isolated mammalian nuclei (5). In the mammalian system, T7 transcription was three orders of magnitude more efficient when naked genomic DNA templates were used, rather than nuclear chromatin preparations. No transcripts could be detected from chromatin templates beyond 82 nt, except at the highest concentration of added polymerase, and in no case could transcripts extending as far as +930 nt be detected. Thus, while promoter-proximal T7 transcripts could be detected, indicating that initiation occurred with some frequency, elongation was inefficient. Flanking the T7 promoter with an immunoglobulin μ enhancer region increased initiation in chromatin by a factor of 30, but had no detectable effect on elongation (5).

Inability of T7 RNAP to elongate templates in higher eukaryotic chromatin would seem a plausible extension of the behavior of the phage polymerase on reconstituted nucleosomal templates *in vitro*. Inhibition of both T7 initiation and elongation by nucleosomes reconstituted from core histones occurs *in vitro* (6–8). More profound inihibition is observed when templates are reconstituted with linker histone H1, in addition to core histones (9). The interpretation that T7 RNAP is unable to negotiate nucleosomal templates is supported by the *in vivo* observation that T7 transcription could not be detected in transgenic *Drosophila*, except in a line bearing a histone H4 mutation or under conditions promoting histone acetylation (4), both of which would be predicted to lead to a more open chromatin conformation (reviewed in 10). Together, these observations reinforced the view that eukaryotic nucleosome and higher order chromatin structure are prohibitive for efficient transcriptional initiation and elongation by T7 RNAP.

Because of the potential utility of a heterologous polymerase in a *trans*-splicing eukaryote and as a prerequisite for the development of an inducible expression system based on a strong promoter, we decided to address the issue of whether T7 RNAP would transcribe chromatin in *Trypanosoma brucei*. Inherent in this organism is a means of circumventing a second problem that has complicated eukaryotic expression systems based on imported T7 transcription, arising from the link between mRNA 5′-cap addition and polymerase (pol) II transcription. Transcripts synthesized in eukaryotic systems by the heterologous polymerase typically fail to receive the 5′-cap structure and are consequently inefficiently translated (11,12). In trypanosomes, the operation of an obligatory *trans*-splicing pathway (reviewed in 13) uncouples transcription and capping, making biosynthesis of viable mRNAs

*To whom correspondence should be addressed. Tel: +1 212 327 7571; Fax: +1 212 327 7845; Email: gamc@rockvax.rockefeller.edu

possible by enzymes other than the endogenous RNA pol II (14,15).

Our initial interest in establishing a T7 transcription system in trypanosomes was a practical one, derived from the lack of a strong inducible promoter which could be insulated from developmental or epigenetic regulation. Inducible expression systems previously described for trypanosomes relied on a tetracycline (Tc)-responsive *parp* promoter (16,17). The *parp* promoter drives expression of the procyclic acidic repetitive protein (PARP; also known as procyclin) genes, encoding the predominant surface proteins of the procyclic (*Tsetse* midgut) form, and is down-regulated in mammalian bloodstream form *T.brucei* (17–20), making it inadequate for strategies requiring high level gene expression. In previous studies, using transgenic trypanosomes (21) or *Leishmania* (22) stably expressing T3 or T7 RNAP, it was shown that these phage polymerases can mediate gene expression from transiently introduced plasmids containing the corresponding promoters. Here, we extend these studies to chromosomally integrated reporters. We find that T7 transcription is highly active in chromatin of *T.brucei in vivo*, possibly reflecting differences in chomatin structure between anciently diverged and modern eukaryotes. We describe a robust inducible expression system founded on the imported T7 RNAP and the Tet repressor (TetR) (23), whose capacity is sufficient for over-expression strategies even in the bloodstream form, where the capacity of previously described inducible expression systems (20) was inadequate.

MATERIALS AND METHODS

Host cell lines and culture conditions

All transgenic cell lines were created in a *T.brucei* strain 427 wild-type background. Construction of procyclic form host cell line 29-13 and bloodstream form host cell line 90-13, co-expressing T7 RNAP and TetR, will be described elsewhere (E.Wirtz, S.Leal, C.Ochatt and G.A.M.Cross, submitted for publication). Procyclic forms were cultured in SDM-79 (24) with 10% fetal calf serum (FCS) at 27°C. Bloodstream forms were cultured in HMI-9 (25) at 37°C. Differentiation of bloodstream form to procyclic form cells was carried out in DTM (26), essentially as described (27).

Reporter constructs

The reporter constructs used are listed in Table 1. Sequence files are available upon request or can be downloaded from www.rockefeller.edu . The starting point for luciferase reporter constructs was pLew18, made by replacing the *Pvu*II–*Sma*I fragment of pHDTop3 (16), encompassing the *parp* promoter and pGEM-derived T7 promoter, with an annealed oligonucleotide pair, 5′-CTGATTAATACGACTCACTATA**G**GGAGATCTCCC-3′ and 5′-GGGAGATCTCCCTATAGTGAGTCGTATTAATCAG-3′, comprising a T7 promoter engineered with a *Bgl*II site at +4. The T7 transcription start site is in bold (**G**) on the coding strand and the *Bgl*II site is underlined. pLew19 was generated from pLew18 by insertion of an annealed oligonucleotide pair comprising a *tet* operator (16) into the *Bgl*II site immediately downstream of the T7 transcription initiation site in pLew18. Mini plasmid preps were sequenced to select single operator insertions orientated in the same direction as the T7 promoter.

Table 1. Summary of constructs for which results are presented

Construct	Figure	Promoters	Operator	Terminator	Markers	
					luc	*ble*
pHD422[a]	1,2	*parp*	–	–	+	–
pLew18	1	T7	–	–	+	–
pLew19	1	T7	+	–	+	–
pLew26	3	T7/*parp*	–/+	–	–	+
pLew27	3	T7/*parp*	–/+	–	+	+
pLew30	3	T7	–	–	+	+
pLew48	1,2	T7	–	+	+	–
pLew49	1,2	T7	+	+	+	–
pLew51	2	T7 G^{-10}	–	+	+	–
pLew52	2	T7 T^{-10}	–	+	+	–
pLew53	2	T7 G^{-10}	+	+	+	–
pLew54	2	T7 T^{-10}	+	+	+	–
pLew55	2	T7 T^{-10}	++	+	+	–
pLew60	4	T7 G^{-10}	+	+	+	+
pLew61	4	T7 T^{-10}	+	+	+	+
pLew82	3–6	T7	+	++	+	+
pLew122	5	*rRNA*	–	–	+	+

[a]Reference 21.

To make a pair of homologs with T7 terminators, the *Pvu*II–*Bam*HI fragment of pLewl8 was exchanged for the same fragment of pHD215, a pHD1 (28) derivative bearing a T7 terminator, synthesized as described previously (21) in the *Pst*I site flanking the *parp* 3′-untranslated region (UTR), giving pLew48. pLew49 was constructed in the same way from pLew19.

To make constructs with T7 promoters of different strengths, for transient transfection assays, pLew51 was made by cloning an annealed oligo pair, EW12 (5′-CCGGTAATACGgCTCACTAT-A**G**GGAGATCTA-3′) and EW13 (5′-CCGGTAGATCTCCCT-ATAGTGAGcCGTATTA-3′, corresponding to a T7 promoter bearing a –10 A→G mutation (T7 G^{-10}), into *Pvu*II–*Bgl*II-digested pLew49, after filling of the 5′ *Age*I overhang, and digestion with *Bgl*II. Again, the T7 start site is in bold, the *Bgl*II site is underlined and the –10 mutation is indicated in lower case. The *tet* operator cassette described above was cloned into the *Bgl*II site of pLew51 to give pLew53. The T7 promoter and luciferase cassette of pLew53 was released as an *Ase*I–*Bam*HI fragment and cloned into the *Asp*718–*Bam*HI site of pLew20, after filling the *Asp*718 overhang, giving pLew57. A T7 terminator was introduced into pLew57 by exchange of its *Sca*I–*Sal*I fragment for that of pHD216 (21), yielding pLew60. pLew62 and pLew63 were generated from pLew60 by insertion of a blunt-ended T7 T^{-10} promoter cassette, bearing a –10 A→T mutation, made by annealing EW14 (5′-CCGGTAATACGtCTCACTATA**G**GGA-GATCTA-3′) and EW15 (5′-CCGGTAGATCTCCCTATAGTG-AGaCGTATTA-3′) and filling the 5′ overhangs, into the *Srf* site. The T7 start is in bold (**G**), the *Bgl*II site is underlined and the –10 mutation is indicated in lower case. pLew52 was made similarly by cloning annealed EW14 and EW15 into *Pvu*II–*Bgl*II-digested pLew49, after filling the 5′ *Age*I overhang, and digestion with *Bgl*II. The *tet* operator cassette described above was cloned into

the *Bgl*II site of pLew52 to give pLew54, containing a single operator, or pLew55, containing two tandem operators.

To make similar reporters containing *ble* to select for stable integration, the *Pvu*II–*Bam*HI fragment of pLew18, containing the T7 promoter and luciferase expression cassette, was cloned into the *Asp*718–*Bam*HI site of pLew20, a close relative of pHD430 (16) in which the pGEM-derived T7 promoter is deleted (E.Wirtz *et al*., submitted for publication), after filling the *Asp*718 overhang, effectively exchanging the *parp* promoter for a T7 promoter and yielding stable reporter pLew30. pLew26 and pLew27 were made by insertion of the T7 promoter oligonucleotide cassette above into the filled *Asp*718 site of pLew5, a linkered version of pLew20 (E.Wirtz, S.Leal, C.Ochatt and G.A.M.Cross, submitted for publication). pLew81 was made by replacement of the *Asp*718–*Bam*HI fragment of pLew20 with the *Pvu*II–*Bam*HI fragment of pLew19. The *Sal*I–*Nhe*I fragment of pLew76 (below) bearing tandem T7 terminators was then cloned into *Sal*I–*Nhe*I-cut pLew81, to give pLew82.

To make stable reporters with T7 promoters of different strengths, the T7 promoter and luciferase cassette of pLew54 and pLew55 were released as *Ase*I–*Bam*HI fragments and cloned into the *Asp*718–*Bam*HI site of pLew20, after filling the *Asp*718 overhang, giving pLew58 and pLew59, respectively. A T7 terminator was introduced into pLew58 by exchange of its *Sca*I–*Sal*I fragment for that of pHD216, yielding pLew61. pLew65 was generated from pLew61 by insertion of a blunt-ended T7 G^{-10} promoter cassette, made by annealing EW12 and EW13 and filling of the 5′ overhangs, into the *Srf* site. A second T7 terminator was inserted in pLew65, by cloning the *Pst*I fragment of pHD216 into the *Apa*I site of pLew65, following blunting of the ends, generating pLew76.

pLew122 was made from pLew50, a derivative of transient luciferase reporter pHD54 (21) containing the rRNA promoter of pLucR (29) in place of the T7 promoter (a replacement of the *Pvu*II–*Xba*I fragment of pHD54 with the *Spe*I–*Xba*I fragment of pLucR, after filling of the *Spe* overhang with Klenow). The *Nde*I–*Xba*I fragment of pLew50 bearing the ribosomal promoter and 5′-region of luciferase was exchanged for the *Asp*718–*Xba*I fragment of pLew20 bearing the *parp* promoter and 5′-region of luciferase, effectively replacing the *parp* promoter of pLew20 with –255 to +38 of the ribosomal promoter of pLew50.

Stable transfections and luciferase assays

For stable transfection of procyclic forms, 29-13 cells, co-expressing T7 RNAP and TetR, or wild-type cells were harvested from a log phase culture ($4–8 \times 10^6$/ml) with a gentle 4°C spin (<700 *g* in a table top centrifuge), washed once in ice-cold Zimmerman post-fusion medium (ZPFM) (30) and resuspended at $2–6 \times 10^7$/ml on ice. Aliquots of $1–3 \times 10^7$ cells in 0.5 ml ZPFM and 5–10 µg DNA were subjected to a single pulse on a BTX electroporator set for peak discharge at 1.6 kV and resistance timing mode R2 (24 Ω). Time constants were typically ~0.3 ms. Cells were then transferred to 10 ml SDM-79 medium with appropriate inhibitors, according to host cell background (G418 at 15 µg/ml plus hygromycin at 50 µg/ml for 29-13 cells; no drugs for wild-type cells) and incubated overnight. Although the presence of inhibitors is not necessary during routine culture of the host cell lines, they are added after transfection to select against transformants in which the new plasmid might displace previous integrations, due to the presence of homologous sequences. Selection for integrated reporters was applied the following day with 2.5 µg/ml phleomycin plus 100 ng/ml Tc for initial induction and selection of transformants. At this time, cells were diluted serially in 24-well microtiter plates, using conditioned medium containing all of the above drugs, to obtain independent clones. Conditioned medium is routinely prepared by pelleting cells from a late log phase culture ($7–9 \times 10^6$/ml) and sterile filtration of the culture supernatant. Following establishment of phleomycin-resistant transformants, phleomycin and Tc were removed by washing the cultures twice in drug-free medium to allow repression of the T7 promoter. Uninduced cultures were maintained in medium containing G418 at 15 µg/ml. For maximum induction of Tc-regulated reporters, Tc was used at 100–500 ng/ml.

For stable transfection of bloodstream forms, 90-13 clone 4 (E.Wirtz, S.Leal, C.Ochatt and G.A.M.Cross, submitted for publication), co-expressing T7 RNAP and TetR, or wild-type cells were harvested from a log phase culture with a gentle room temperature spin (<700 *g* in a table top centrifuge), washed once in Cytomix (31) at 37°C and resuspended in Cytomix at 2×10^7/ml. Aliquots of 1×10^7 cells and 10 µg DNA were electroporated as for the procyclic form. Following electroporation, cells were transferred to 10 ml HMI-9 and survivors counted. Microtiter plates (24-well) were seeded with $3–10 \times 10^4$ living cells/well in a volume of 0.5–1 ml. Selection was applied the following day by adding the same volume of HMI-9 containing a 2-fold drug concentration for a final concentration of 2.5 µg/ml phleomycin and 100 ng/ml Tc, in addition to 5 µg/ml hygromycin and 2.5 µg/ml G418, to ensure retention of the T7 RNAP and TetR transgenes. Following establishment of phleomycin-resistant clones, phleomycin and Tc were withdrawn by washing the cultures twice in drug-free medium. Uninduced cultures were maintained in medium containing G418 at 2.5 µg/ml. For maximum induction, Tc was used at 100–500 ng/ml.

For luciferase assays from stable expressers, a Promega luciferase assay system was used. Aliquots of 2×10^6 cells were gently pelleted and lysed with 100 µl Promega lysis buffer (or 0.2% Triton in phosphate-buffered saline) on ice. Cell debris was removed by a 3–5 min microfuge spin at 4°C. Samples of 5 µl cleared supernatant were added to 45 µl Promega assay buffer containing luciferin, at room temperature, and samples were read immediately in a Turner model TD-20e luminometer. All stable cell lines were assayed in duplicate multiple times and results from representative experiments are shown.

Transient transfections and luciferase assays

Samples of $1–2 \times 10^7$ cells were transfected with 5–10 µg DNA, using the electroporation conditions described above, then transferred to 5 ml medium and incubated for 12–20 h. For luciferase assays the entire culture was harvested (gentle table top spin at 4°C). Cells were lysed and assayed as described above. Transient transfections were done at least twice and assayed in duplicate.

Regulation during animal infection

Mouse infections were established by i.p. inoculation of 10^6 bloodstream form trypanosomes derived from culture. For induction, 200 µg/ml doxycycline were administered to mice in their drinking water with 5% sucrose (32), 1 week prior to infection. Uninduced controls were given 5% sucrose without doxycycline. Trypanosomes were harvested from blood when

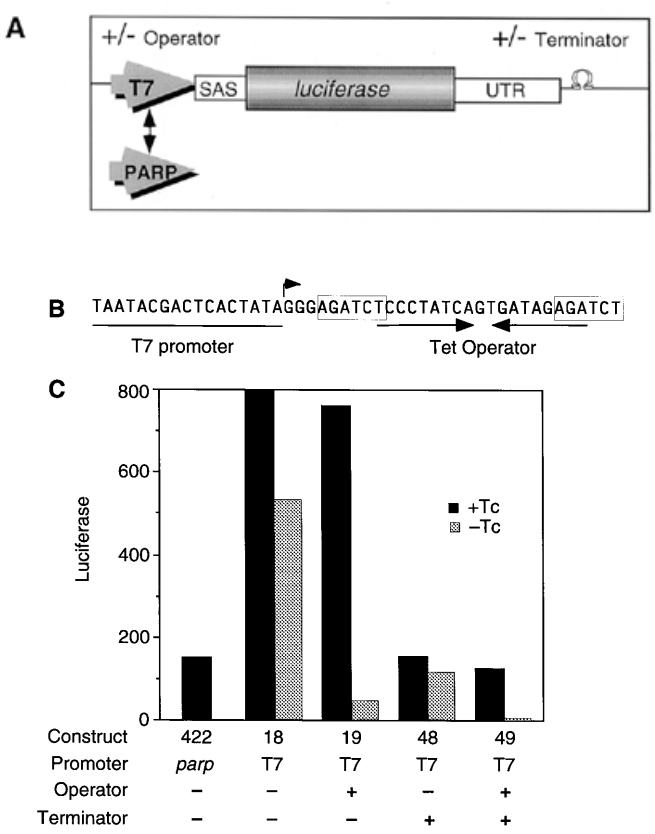

Figure 1. Regulation of the T7 promoter by TetR in transient assays. (**A**) Transient reporter constructs shared a modular design in which a T7 or a *parp* promoter plus or minus a *tet* operator were situated to drive expression of a luciferase cassette flanked by 5′ and 3′ RNA processing signals plus or minus a downstream T7 terminator. SAS, region supplying signals for capped spliced leader RNA addition. UTR, 3′-untranslated region supplying the poly(A) addition site. Relevant regions of reporters are shown schematically in linear form although supercoiled plasmid was used for transfection. (**B**) *tet* operator insertion in the T7 promoter. The T7 transcription initiation start site is indicated. Arrows indicate operator half-sites. *Bgl*II sites used for operator insertion are boxed. (**C**) Regulation of the T7 promoter in transient assays. Aliquots of 5 µg supercoiled plasmid DNA were used to transfect 10^7 procyclic forms of the 29-13 line. Luciferase activities are given as light units per 10^5 cells. Transient luciferase activity from a *parp* promoter (pHD422; 21) is shown for comparison.

parasitemia reached ~10^9 trypanosomes/ml and assayed for luciferase activity.

RESULTS

Engineering a tetracycline-responsive T7 promoter

The feasibility of creating a T7 RNAP-based inducible expression system depended upon two critical issues: whether this polymerase could efficiently transcribe chromatin in *T.brucei* and whether a regulatable T7 promoter could be engineered for trypanosomes. We first investigated whether the T7 promoter could be regulated by TetR (23). Reporter plasmids (Fig. 1A) were constructed with luciferase expression cassettes linked to a wild-type T7 promoter or one containing a *tet* operator insertion at +9 (Fig. 1B). These were transiently transfected in the presence or absence of Tc into a procyclic cell line (29-13) stably co-expressing T7 RNAP and TetR (E.Wirtz, S.Leal, C.Ochatt and G.A.M.Cross, submitted for publication) and luciferase was assayed (Fig. 1C). The wild-type T7 promoter mediated high level luciferase expression, surpassing that mediated by the *parp* promoter by >5-fold, in agreement with previously reported transient activity of T7 promoters (21). Similarly, the T7 promoter containing a *tet* operator generated high levels of luciferase, but also showed responsiveness to Tc in this background of TetR expression. In these initial transient assays the regulation factor was only 10-fold, largely due, we surmised, to run-around transcription on the circular templates. When reporter plasmids were re-engineered to contain a T7 terminator (33) downstream of the luciferase 3′-UTR, maximum expression was reduced, confirming a contribution of run-around transcription. More importantly, background expression in the absence of Tc fell, increasing the regulation factor to >50-fold and pointing to the potential of a Tc-responsive T7 promoter as the basis for an inducible expression system.

Activities of T7 promoter mutants of different intrinsic strength

Concise intermediate strength promoters, such as those potentially involved in pol II transcription, have not yet been defined in trypanosones. In addition to the strong inducible promoter represented by the full-strength Tc-responsive T7 promoter, we sought to generate constitutive promoters of intermediate intrinsic strength, to drive expression of introduced genes at levels approximating those mediated by endogenous pol II. We introduced point mutations in the wild-type T7 promoter, 10 nt upstream from the start site (Fig. 2A), and measured the activity of these mutated promoters relative to the *parp* and wild-type T7 promoters in transient assays in procyclic forms expressing T7 RNAP (Fig. 2B). Qualitatively, the effect of these mutations paralleled what has been reported for the same mutations in an *in vitro* transcription system (34). The T^{-10} transition reduced activity 10-fold; the G^{-10} transversion ~400-fold.

The ability to regulate the point mutant T7 promoters with TetR was checked by inserting one or two operators into both the T^{-10} and G^{-10} promoters. In transient assays in the presence or absence of Tc, the T^{-10} promoter yielded a regulation factor of ~100-fold, while the weaker G^{-10} mutant showed <2-fold regulation, when considered in the light of the control construct without an operator.

Stable reporters for T7 transcription in the chromosome: lethality of T7-mediated antisense transcription in rDNA and rescue by termination

In order to test the ability of T7 to initiate and elongate chromatin templates in *T.brucei*, we constructed bicistronic stable reporters consisting of a luciferase reporter cassette linked to a selectable marker, both under the control of a T7 promoter. The first of these stable reporters, pLew30, consisted of a derivative of inducible expression vector pHD430 (16), in which a wild-type T7 promoter replaced the *parp* promoter (Fig. 3). This construct was designed for homologous targeting to a transcriptionally silent intergenic spacer of the *T.brucei* rDNA repeat (35), where the reporter cassette is insulated from read-through transcription by endogenous polymerases (16). pLew30 was linearized and used to transfect T7 RNAP-expressing procyclic forms. Repeated attempts to obtain phleomycin-resistant transformants with this construct resulted in transient emergence of weakly resistant

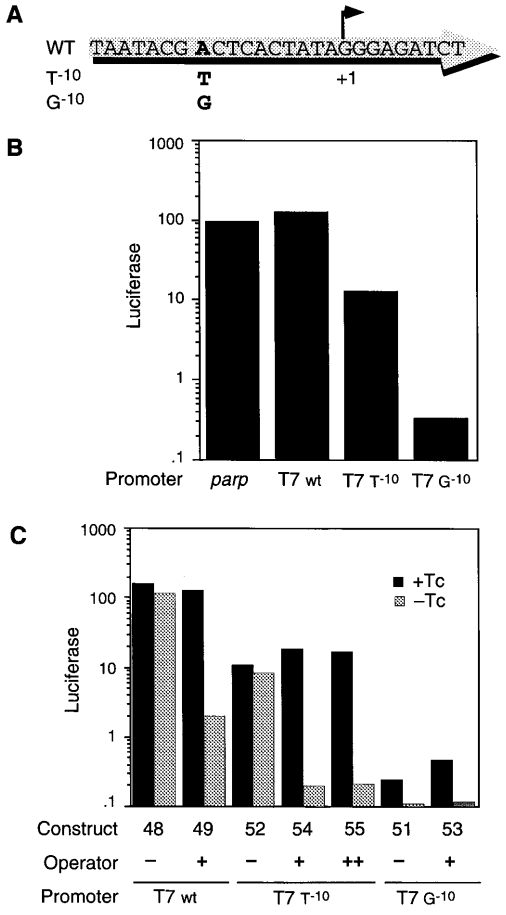

Figure 2. Generating T7 promoters of different relative strength. (**A**) T7 promoter mutations. (**B**) Activities of unregulated promoters in transient assays. pLew48 (wild-type T7 promoter), pLew52 (T^{-10} T7 promoter) or pLew51 (G^{-10} T7 promoter) were used to transfect 29-13 procyclic forms. Reporter anatomy is as shown in Figure 1, except for the absence of operator insertions. For *parp* promoter activity, pHD422 (21) was used. (**C**) Tc regulation of T7 promoters in transient assays. 29-13 procyclic form cells were transiently transfected with the indicated constructs and cultured overnight in the presence or absence of Tc (500 ng/ml). Luciferase activities are given as light units per 10^5 cells. The low induced and uninduced levels shown by the G^{-10} mutant fall within the range of variation intrinsic to transient transfection assays with these cells. The instrument background was <0.005 light units.

clones. These were inviable upon expansion, consistent with a prediction from other eukaryotic systems that T7 transcription in *T.brucei* chromatin would not be processive. However, we explored an alternative explanation.

In pLew30, transcription of the integrated cassettes opposes that of the endogenous rRNA promoter, as part of the original design, which was intended to eliminate transcriptional read-through of the reporter. Although there had been no prior indication that antisense transcription directed by trypanosome *parp*, *rRNA* or *vsg* expression site promoters in rDNA was problematical (16,17,20), we hypothesized that antisense transcription mediated by a highly processive phage polymerase might have lethal consequences. Our ability to readily obtain transformants using the pLew30 relatives pLew26 and pLew27 (Fig. 3), in which the orientation of the T7 promoter is the same as that of the endogenous 18S *rRNA* promoter, was consistent with this hypothesis. We therefore constructed pLew82, in which dual T7 terminators were placed immediately downstream of the *ble* cassette. In pLew82, the wild-type T7 promoter of pLew30 was also exchanged for the *tet* operator-containing T7 promoter, to expedite development of inducible integration vectors. When pLew82 was transfected into the 29-13 procyclic form host in the presence of Tc, but not in a wild-type background, phleomycin-resistant transformants were obtained at high frequency and grew out of selection vigorously. The robust resistance suggested that T7 was highly active on chromatin templates, implying that lethality of antisense T7 transcription in the rDNA was the problem with pLew30. The anatomy of these reporters, where the distance from the transcription start site to the poly(A) addition site of the *ble* cassette is ~3 kb, pointed to an unprecedented processivity for T7 transcription in eukaryotic chromatin.

Regulation of different Tc-responsive T7 promoters in the chromosome

The ability to terminate T7 transcription in chromatin enabled us to ascertain regulation characteristics and transcription levels mediated by the different Tc-responsive T7 promoters in a chromosomal context. Derivatives of pLew82, containing the Tc-responsive promoters of pLew51–55 (Fig. 2), were used to transfect T7 RNAP/TetR-expressing procyclic form trypanosomes. Phleomycin-resistant transformants were selected in the presence of Tc, to ensure activity of the T7 promoter, which was necessary for selectable marker expression. After establishing phleomycin-resistant clones, Tc and phleomycin were withdrawn and clones were maintained in the absence of Tc to permit repression of T7 transcription. Luciferase was subsequently assayed in cells maintained in the presence or absence of Tc (Fig. 4). Luciferase levels mediated by endogenous pol I or pol II are shown for comparison. Results are shown for two pol I promoters, integrated into the *rRNA* spacer in exactly the same context as the T7 promoter of pLew82. One is the Tc-inducible *parp* promoter of pLew20 (E.Wirtz, S.Leal, C.Ochatt and G.A.M.Cross, submitted for publication), a close relative of published inducible vector pHD430 (16). The second is a *T.brucei rRNA* promoter (15,36,37). Pol II is represented by transcription of a promoterless luciferase cassette inserted into the *tubulin* locus (16).

The full-strength T7 promoter drove luciferase activity to higher levels than those attained with the *rRNA* or *parp* promoters, in a Tc-dependent manner. This T7 promoter could be regulated through a 200-fold range in procyclic forms, with maximum expression 2- to 5-fold higher than *parp* promoter levels and background expression at ~20% of pol II read-through levels. The T^{-10} and G^{-10} promoter mutants afforded <10-fold regulation. Maximum expression levels for the two mutant promoters hovered just above or just below the range of reporter activity driven by pol II read-through, respectively. We have exploited these weaker constitutive T7 promoters, of pol II-like strength, in applications that are described in detail elsewhere (E.Wirtz, S.Leal, C.Ochatt and G.A.M.Cross, submitted for publication).

Induction and repression kinetics of the Tc-dependent T7 promoter

pLew82 was subsequently transfected into T7 RNAP/TetR-expressing bloodstream forms (cell line 90-13; E.Wirtz *et al.*,

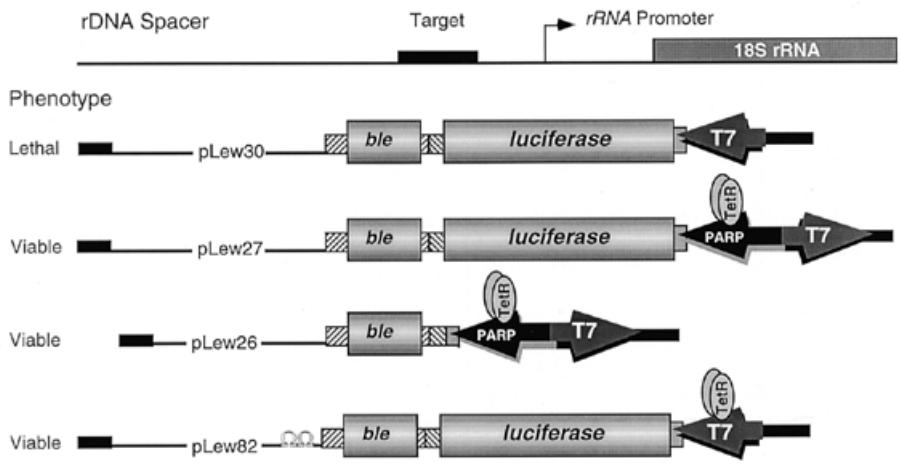

Figure 3. Toxicity of antisense transcription in rDNA. Anatomy of four stable reporters used for transfection of wild-type or transgenic 29-13 trypanosomes. Constructs were designed for homologous targeting to the rDNA non-transcribed spacer. Constructs are shown schematically in the linear form used for stable transfection. Solid black boxes indicate extent of homology to rDNA spacer. Untranslated regions supplying RNA processing signals flanking *luc* and *ble* are derived from *T.brucei parp* (open box), *aldolase* (left hatching) or *actin* (right hatching) loci. Promoter regions are shown as large arrowheads and terminator regions as hairpins. Plasmid backbones are shown as a thin black line. Constructs that failed to yield viable transformants in more than three independent positively controlled transfections (9×10^7 cells) were scored as lethal.

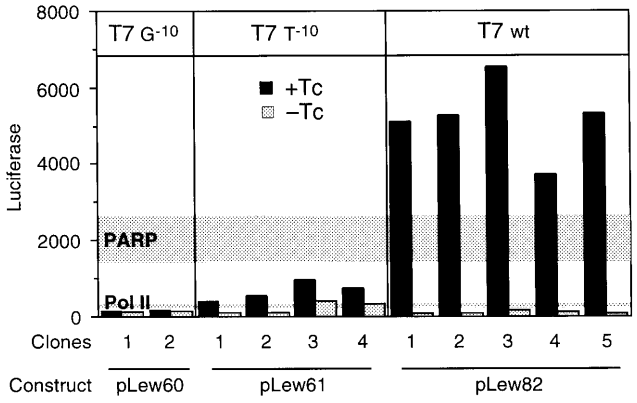

Figure 4. Regulation of T7 promoters of different strengths in the chromosome. Aliquots of 10 µg *Not*I-linearized pLew60, pLew61 or pLew82 were used for stable transfection of procyclic form cell line 29-13, as described in Materials and Methods. Independent clones were assayed for luciferase activity under inducing and non-inducing conditions. Following establishment of phleomycin-resistant transformants, phleomycin and Tc were removed by washing cultures twice in drug-free medium, to allow repression of the T7 promoter. Uninduced cultures were maintained in the absence of Tc for ~1 week before induction with 500 ng/ml Tc. Luciferase activities for 10^5 cells were determined ~48 h after induction. The horizontal shaded bars indicate the range of mean luciferase activities driven by a *parp* promoter in the same context (pLew20) (E.Wirtz *et al*., submitted for publication) and the range of mean luciferase activities obtained from six independent pHD421 (16) procyclic form transformants assayed multiple times over several months, representing pol II read-through at the *tubulin* locus.

submitted for publication). Phleomycin-resistant transformants were routinely selected in the presence of 100 ng/ml Tc. We found, however, that bloodstream and procyclic transformants could be selected with as little as 1 ng/ml Tc. This permits the introduction of genes, under the control of the inducible T7 promoter, that are toxic when expressed at a high level. As for the procyclic form, no transformants could be obtained when T7 transcription was unterminated. In cultured bloodstream forms, transformants showed regulation factors in the range 200- to 1000-fold, owing to lower background expression (Fig. 5 and data not shown). Maximum expression of luciferase in the bloodstream form was also lower, which was attributed to several factors, including reduced stability of luciferase in bloodstream form cell lysates (E.Wirtz, S.Leal, C.Ochatt and G.A.M.Cross, submitted for publication) and thermolability of the enzyme (38,39) at the culture temperature (37°C) of the bloodstream form. As in procyclic forms, background expression was ~20% of pol II read-through for cultured bloodstream forms, but lower in animals (see below).

We went on to determine induction kinetics for the T7-based vector in the two developmental stages. Kinetics for full-strength T7 promoters in bloodstream form and procyclic *T.brucei* are presented in Figure 5. The T7 promoter in the bloodstream form (Fig. 5A) exhibits rapid induction kinetics. Maximum expression levels, within or exceeding the range of expression mediated by an *rRNA* promoter in the same context, were reached in 1–2 h. Within 15 min, levels were in the range of the developmentally attenuated *parp* promoter and maximum induction was ~20-fold higher. We took advantage of the rapid turnover of luciferase in bloodstream forms to examine repression kinetics. When Tc was withdrawn after a period of 48 h of maximum induction, baseline luciferase levels were reached within 5 h. We were unable to accelerate switch-off kinetics using the Tc antagonist GR33076X (40). Induction kinetics were somewhat slower in the procyclic form, whose doubling time is about twice that of the bloodstream form. Expression levels equivalent to those driven by the *parp* promoter were reached within 4 h and expression equivalent to pol II read-through was reached within 1 h.

The outcome of these experiments is a robust inducible promoter for high level expression, particularly needed in the bloodstream form, where the *parp* promoter is relatively weak due to developmental down-regulation.

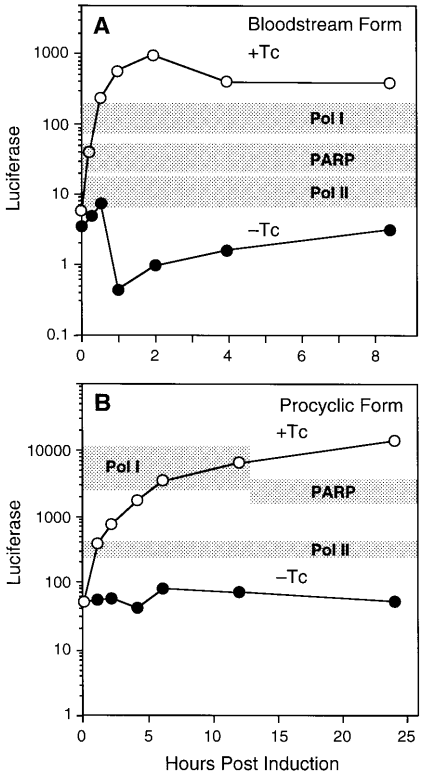

Figure 5. Induction kinetics for a Tc-responsive T7 promoter in representative bloodstream form (**A**) and procyclic form (**B**) clones. Luciferase activities from 10^5 cells are plotted on a logarithmic scale to reveal background expression in the absence of Tc. Horizontal shaded bars represent the range of mean luciferase activities obtained from: pol II, three (bloodstream form) or six (procyclic) independent pHD421 (16) clones; pol I, three independent pLew122 (*rRNA* promoter) bloodstream form clones or derived procyclic form clones; PARP, multiple pLew20 (*parp* promoter) transformants. The pHD421 (pol II) and pLew20 clones were obtained by direct transfection of procyclic or bloodstream forms, while the three pLew122 procyclic form clones (pol I) were derived by differentiation of the bloodstream form transformants.

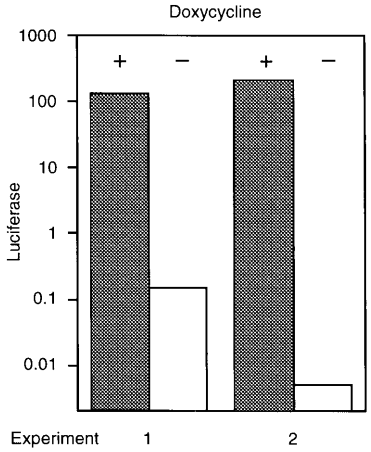

Figure 6. Regulation of T7-mediated expression during animal infection. Luciferase expression was induced in bloodstream forms (pLew82-90-13 clone 2) during infection in mice by administration of 200 µg/ml doxycycline in drinking water. Luciferase activity is given as light units per 10^5 trypanosomes purified from blood of induced or uninduced mice at peak parasitemia (10^9 trypanosomes/ml). Two animals were used in each experiment.

Regulation of gene expression during animal infections

In the pathogenic trypanosomes, where gene function questions often concern aspects of the host–parasite interaction, the value of a regulatory system depends to a great extent on its ability to function during animal infection. Mouse infections were established using a 90-13 bloodstream form clone containing the pLew82 integration. Luciferase activities were determined for trypanosomes isolated from mice supplied with doxycycline in their drinking water (Fig. 6). Trypanosomes from infected animals receiving doxycycline yielded luciferase activities 1000- to 10 000-fold higher than those from uninduced animals. The maximum induced level of expression in animals mirrored levels observed in culture, while uninduced levels were lower. Background levels corresponded to 0.4–3 luciferase molecules/5 cells, calculated from standard luciferase assays in which purified luciferase was added to trypanosome lysates.

DISCUSSION

The very limited availability of characterized genetic regulatory elements, including promoter and terminator elements, in trypanosomes provided the impetus for establishing transgenic *T.brucei* lines stably expressing T7 RNAP (21). The activity of the phage polymerase in the original cell lines, however, was tested only on transiently introduced reporter plasmids. We expanded those studies, to examine the behavior of the phage polymerase on chromatin templates *in vivo*, as a prerequisite to using it as an inducible expression system. An inducible Tc-responsive T7 promoter was engineered on the basis of transient transfection of reporters into trypanosomes co-expressing T7 RNAP and TetR and its capacity to transcribe chromatin templates was investigated using chromosomally integrated reporters. In developing a strong heterologous inducible promoter for trypanosomes, we found that the behavior of T7 RNAP in trypanosome chromatin differs in some surprising and perhaps instructive ways from its behavior in other eukaryotic systems. The ability of T7 RNAP to vigorously transcribe trypanosome chromatin is unprecedented in eukaryotes, either *in vivo* or on templates reconstituted from mammalian histones. The interest of this result resides both in the methodological gains it translates into, in the form of a robust inducible expression system for a lower eukaryotic system, represented by *T.brucei*, and in its implications for chromatin structure in this organism.

The fact that chromatin structure at these rDNA integration sites in trypanosomes is less inhibitory for T7 transcription than prior work with higher eukaryotic transcription systems would have predicted could reflect general features of trypanosome chromatin and/or a local feature of rDNA chromatin structure. Global structural differences responsible for a generally enhanced accessibility could map to the intrinsic nucleosome or chromatosome unit, at the level of the core or linker H1 histones, respectively. Regional differences would be expected to map to higher order structure, governed by locus control elements, influencing the transcriptional status of large chromatin domains, such as those encompassing rDNA.

A number of observations support the idea of globally heightened accessibility. Trypanosome chromatin is more accessible to micrococcal nuclease digestion than higher eukaryotic chromatin,

suggesting a more open conformation (41). Trypanosome chromosomes do not visibly condense during metaphase (42) and isolated chromatin shows a different compaction pattern *in vitro* from that of higher eukaryotes. Nucleosome filaments, while they do form under some conditions, do not undergo the degree of condensation seen in higher eukaryotes to form solenoids (43), perhaps due to the divergent H1 histones and modifications (44,45). However, some inhibition of T7 RNAP-mediated transcription can be measured from templates reconstituted with higher eukaryotic core histones alone, in the absence of linker histones. These results potentially implicate the trypanosome core nucleosome as one basis for the greater template accessibility seen *in vivo*, barring an artifact of the *in vitro* transcription systems such as promotion of condensation by the high salt conditions. Available trypanosome core histone sequences do show small but significant differences from their higher eukaryotic counterparts (46).

In addition to the implication that trypanosome chromatin is generally more open than that of higher eukaryotes, there may be an especially accessible chromatin structure within the rDNA of the procyclic form. There is evidence that *rRNA* transcription units exist in an open accessible chromatin conformation in higher eukaryotes. The subset of *rRNA* genes that are actively transcribed in yeast, as well as their associated non-transcribed spacer 3′ enhancer, have been shown to exist in a non-nucleosomal conformation (47,48). Existence of procyclic form rDNA in a specialized non-nucleosomal structure would provide a partial explanation for the pronounced accessibility of reporters in this developmental stage. However, we have also observed processive transcription of T7 reporters integrated in non-rDNA housekeeping loci transcribed by pol II, albeit at a significantly lower level (M.Navarro, G.A.M.Cross and E.Wirtz, manuscript in preparation). As to whether our approach selects for integration at non-nucleosomal spacers, the fact that transcription of our cassettes can be induced to high levels months after removal of drug pressure argues against this, if the transcriptional status of trypanosome rDNA is metastable and non-heritable, as it is in yeast (48). In contrast to procyclic forms, T7 RNAP-mediated expression from rDNA in bloodstream forms was no higher than expression from T7 promoter integrations into pol II-transcribed loci. This raises the possibility that rDNA loci of the bloodstream form are targeted by a form of regulation, akin to that giving rise to the rDNA position effect recently described in yeast (49,50), that is absent from procyclic forms. It seems plausible, therefore, that the vigorous T7 transcription of trypanosome chromatin partly reflects a specialized rDNA structure, as well as some fundamental difference in chromatin structure between trypanosomes and higher eukaryotes.

These results raise the possibility that the T7 RNAP–promoter interaction could be used as a probe for chromatin accessibility, in order to define *cis*-acting elements governing the transcriptional status of chromatin domains. The interaction of the single subunit polymerase with its cognate promoter has been used in higher eukaryotic systems as a reporter for *cis* elements mediating chromatin accessibility (4,5,51). In this application, the trypanosome system affords certain technical advantages. The two primary limitations that have constrained development of T7 RNAP-based protein expression technologies in other eukaryotic systems do not apply to trypanosomes, where *trans*-splicing supplies the 5′ mRNA cap and processive transcription of chromatin is not a problem. A very sensitive and simple enzyme assay can be used as a reporter for T7 transcription, obviating the need to rely on primer extension or other methods of RNA analysis to detect short, low abundance T7 transcripts, as is required for other eukaryotic systems. This permits a totally *in vivo* analysis of the accessibility of a given template to the small polymerase. An *in vivo* approach represents a significantly diminished disturbance, compared with analyses based on isolation of nuclei or chromatin, and could potentially yield a more authentic picture of the constellation of protein–DNA interactions linked to the control of a given locus. We are currently exploring the use of chromosomally integrated T7 promoters, with linked luciferase reporters, to investigate sequence requirements of epigenetic control mechanisms targeting developmentally regulated loci in trypanosomes. It is conceivable, in this eukaryotic system marked by a conspicuous absence of regulation of pol II transcription at the level of initiation and absence of concise pol II promoters, that transcription is largely controlled by template accessibility.

Whatever its implications or utility for probing chromatin structure in trypanosomes, the vigorous T7 transcription, the ability to control it with Tc, to terminate it and to set different levels of constitutive expression with promoter mutations, have allowed versatile inducible expression systems to be developed for trypanosomes. The ability to engineer a Tc-responsive T7 promoter circumvents the endogenous regulation that is superimposed on the previously described Tc-responsive *parp* promoter (16). Although regulation of the T7 promoter with the *lac* repressor has been described (52), repression of T7 transcription by the much smaller TetR complex has not been reported.

An incidental finding, revealed by the failure of unterminated rDNA targeting vectors to yield viable transformants, was that antisense T7 transcription in the rDNA locus is lethal. This effect could be caused by antisense or dsRNA targeting rRNA biosynthesis *in trans* or by the disruption *in cis* of protein–DNA interactions involved in replication or regulation of the rDNA locus. In other systems, many such *cis*/*trans* interactions map within the rDNA intergenic spacer region (53). We now routinely include terminators to prevent T7 RNAP read-through into flanking genes.

While the T7 system was designed and is clearly suited for reverse genetic strategies requiring high level expression, repressed levels amounted to ~20% of pol II read-through, in both procyclic and bloodstream form cultures. Regulation was significantly tighter in animals, where induction factors exceeded 3×10^4. Although leaky regulation will not present a problem for many dominant-negative approaches, it may be unsuitable for expressing highly toxic products. To address this situation, we have developed a dual promoter vector with an inducible *parp* promoter and constitutive T7 promoter, which exhibits extremely low background expression (E.Wirtz, S.Leal, C.Ochatt and G.A.M.Cross, submitted for publication).

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