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A low-background inducible promoter system in *Leishmania donovani*

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Abstract

We report here a second-generation tetracycline-responsive repressor–operator system in *Leishmania donovani*. In this system, expression of a reporter luciferase gene (*LUC*) is driven by the inducible *Leishmania* ribosomal RNA promoter on the DNA strand opposite to a hygromycin resistance gene (*HYG*) whose expression is driven by the endogenous pol I promoter on chromosome 27 (*rDNA* locus) or the endogenous pol II promoter on chromosome 35 (LD1 locus). Transgenic cell lines showed regulation of *LUC* gene expression over three orders of magnitude. In the absence of tetracycline, luciferase expression levels were 2–3-fold higher than machine background when integrated into the LD1 locus, but was over 10-fold higher than machine background when integrated into the *rDNA* locus.

Keywords: Hygromycin resistance gene; LD1 locus; *Leishmania donovani*; Luciferase gene

1. Introduction

Leishmania are flagellated protozoa that are transmitted to mammals by the bite of an infected sandfly. They cause a spectrum of human diseases, ranging from localized cutaneous lesions to lethal disease, in tropical and subtropical regions [1]. Despite their importance to public health, little is known about the molecular biology of *Leishmania*, including their transcription processes. What is known in *Leishmania* is that protein-coding genes are transcribed as polycistronic precursor RNAs which are subsequently processed into single gene mRNAs by 3′ polyadenylation and 5′ trans-splicing [2]. The apparent absence of untranscribed intergenic regions suggests that RNA polymerase II (pol II) transcription in *Leishmania* differs from that of higher eukaryotes. Attempts to identify pol II promoters have been problematic and have not yet led to the characterization of a bona fide pol II promoter [3]. However, RNA polymerase I (pol I) promoters have recently been identified in the *rDNA* locus of *Leishmania chagasi* [4], *Leishmania amazonensis* [5] and *Leishmania donovani* [6]. Interestingly, the transcription of protein-coding genes can be sometimes driven by a pol I promoter [5–7].

We have reported the adaptation of the tetracycline-responsive repressor–operator system for use in *L. donovani* [8]. In this system, the tetracycline-responsive repressor (TETR) binds to the tetracycline-responsive operator (*TetO*) close to the transcription initiation site (TIS) and prevents transcription [9]. Transcription is induced by addition of tetracycline, which binds to the repressor, causing it to dissociate from the operator, thereby allowing transcription to proceed. The first-generation system contains a bleomycin resistance–luciferase fusion (*BLE-LUC*) gene driven by a ribosomal RNA promoter (P_{RRNA}) with two copies of the operator sequence inserted two nucleotides upstream of the

Abbreviations: bp, base pair; kb, kilobase pair; nt, nucleotide; pol I, RNA polymerase I; pol II, RNA polymerase II; P_{RRNA}, ribosomal RNA promoter; rRNA, ribosomal RNA; TIS, transcription initiation site; TETR, tetracycline-responsive repressor; TetO, tetracycline-responsive operator.

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TIS [8]. When targeted into the *rDNA* locus on chromosome 27 (Chr27) of *L. donovani* in the reverse orientation relative to transcription of the *rDNA* genes, this system allowed regulation of *BLE-LUC* expression by two orders of magnitude. The expression level in the absence of tetracycline was ~50-fold lower than that of the *BLE-LUC* gene in the tubulin locus (where it is transcribed by RNA polymerase II), while the expression level in the presence of tetracycline was ~5-fold higher than that from the tubulin locus. This system allows substantial up- or down-regulation expression of endogenous or exogenous genes. However, expression of the selectable marker in this construct also depends on the induction of the promoter. This co-regulation of the selectable marker and test gene is not suitable in many cases, e.g. over-expression of toxic gene products. In addition, background expression levels (in the absence of tetracycline) are still 100-fold higher than machine background. It is unknown whether this background is due to read-through transcription in the *rDNA* locus, and/or a leaky promoter. In addition, since the *BLE-LUC* gene was used for both selection and as a reporter, mutants that result in the high background may have been inadvertently selected. Thus, to improve the inducible system, we developed a second-generation inducible promoter system in which the expression of test gene (*LUC*) and a different selectable marker, the hygromycin resistance gene (*HYG*) are on opposite DNA strands and driven by different promoters.

2. Materials and methods

2.1. Parasite cell culture

All parasite cell lines used in this study were derived from *L. donovani* MHOM/SD/00/Khartoum (LSB-51.1), which contains an additional copy of *ORFF* and *BT1* in the *rDNA* locus, as a result of gene conversion from the LD1 locus on chromosome 35 [7]. Promastigotes were grown at 24 °C in supplemented AM medium, as described previously [7].

2.2. Plasmid constructs

Construct pGUH1 (Fig. 1) was designed to place the selectable marker (*HYG*) under the control of the P_{RRNA}, downstream from the *LUC* gene. The 5′ targeting sequence, part of the *BT1* (formerly *ORFG*) coding sequence, was obtained from *Apa*I + *Kpn*I-digested plasmid K27 [7] and ligated to *Apa*I + *Kpn*I-digested pBluescript II SK$^-$ to produce pG. The 3′ target sequence was PCR amplified from pHE20 [7] using primers 5′SBSTER (5′AGTCA*CCGCGGCCAATGCATTGG*TGGAGGTGGGAATGGG3′) and 3′SNTER (5′AGTCA*CCGCGGCTAGC*GGATGTCGAATTCCGAATGTG3′). This PCR product was digested with *Sac*II and ligated with *Sac*II-digested pG to produce pGT. The *HYG* gene (along with the 5′ and 3′ flanking sequences) was obtained by *Sal*I + *Ssp*I digestion of pX63hyg [10], followed by filling in with Klenow, then

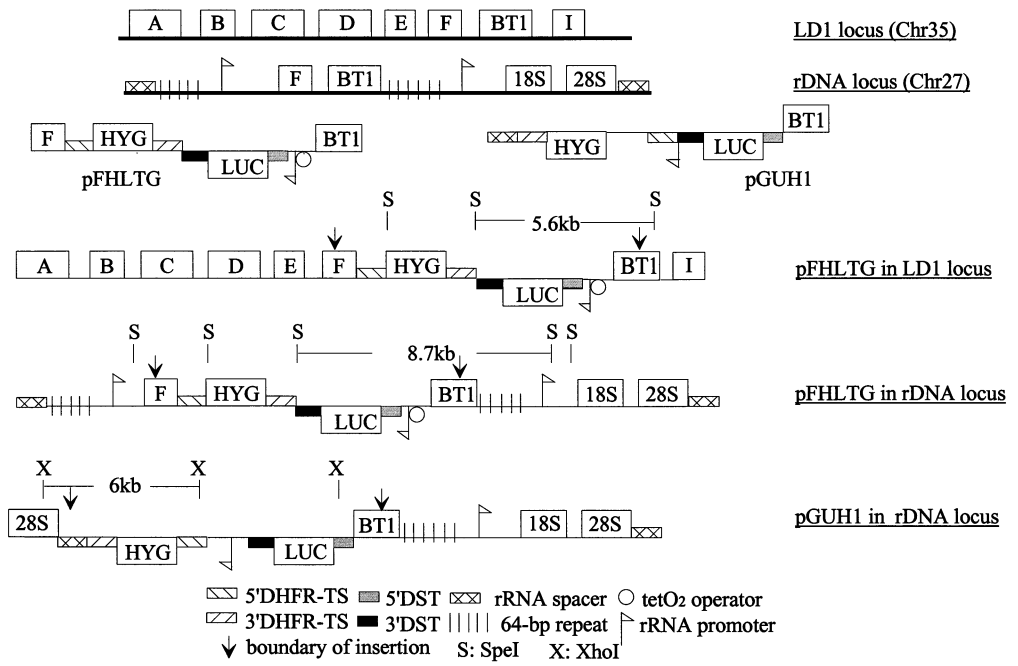

Fig. 1. Schematic depiction of constructs used for stable transfection of *Leishmania*. pFHLG is similar to pFHLTG except that the inducible promoter was deleted by restriction digestion and religation. The arrows represent sites of targeting for integration into the genome. Genes are represented by boxes whose position indicates their coding strand and flanking sequences are indicated by shaded boxes.

digestion by *Nsi*I. The resultant 3.1 kb fragment was ligated into *Eco*RV + *Pst*I-digested pGT to generate pGTH. The *LUC* gene was PCR-amplified from pLUH207 [11] using primers 5′SmBgluc (5′TGATG*CCCGGGAGATCT*TATGGAAGACGCCAAAAAC3′) and 3′BamAvluc (5′CTGCT*GGATCCTAGG*ATTCGATATTTTTCCATAATTTTCTTG3′). This PCR product was digested with *Sma*I and *Bam*HI and ligated into *Sma*I + *Bam*HI-digested pxG [12] to produce pxGpLuc. In this plasmid the processing signals of *LUC* were derived from the *DHFR-TS*/*DST* intergenic region [13]. pxGpLuc was digested with *Nsi*I, blunt ended by Klenow, then digested with *Pvu*I, *Xho*I and *Hin*dIII to generate a 3 kb fragment containing *LUC* with its 5′ and 3′ flanking sequences. This fragment was ligated to the fragment resulting from the *Cla*I digestion, filling in by Klenow, and *Hin*dIII-digestion of pGTH to produce pGLH. The promoter sequence was produced by *Hin*dIII digestion of a PCR product amplified from pE45 [6] using primers 5′HdBprom (5′CACT*AAGCTTGGATCC*TGTGAGTTATGAGGTCTGCG3′) and 3′Hdprom (5′CACT*AAGCTT*CACAAACACG GCATCCAC3′), and ligation to *Hin*dIII-digested pGLH, to generate pGUH1.

pFHLTG (Fig. 1) was designed to insert the *LUC* gene under the control of a tetracycline-inducible P_{RRNA}, downstream, and on the opposite strand, of *HYG*, which is driven by the endogenous pol I or pol II promoter within the *rDNA* or LD1 locus. The inducible promoter sequence (in which the TetO sequence was integrated two nt upstream of the TIS) was PCR-amplified from pGTBUC [8] using primers 151prom (5′ACT*GGGCCC*TGTGAGTTATGAGGTCTGCG3′) and 3′salprom (5′ACGCAT*GTCGAC*ACAAACACG GCATCCAC3′). The *Apa*I + *Sal*I-digested PCR product was then ligated into *Apa*I + *Sal*I-digested pGLH to generate pGTLH. The 5′ targeting sequence was PCR-amplified from pE45 using primers 5′BsF (5′CTGCAGAA*CCAATGCATTGG*AACTGCTGTCGGCTGTATGCTC3′) and 3′NhAsF (5′GCAGAAG*CTAGCGGCGCGCC*ACCACGC TCCACAATTTCG3′). The gel-purified PCR product was digested with *Bst*XI and *Nhe*I and ligated with *Bst*XI + *Nhe*I-digested pGTLH to generate pGTLHF. The orientation of the *HYG* gene was reversed by digestion of pGTLHF with *Hin*dIII and *Nhe*I to generate 8.1 and 3.2 kb fragments, which were blunt-ended using Klenow and religated. The resultant clones were screened by restriction digestion for the final construct pFHLTG, in which the *HYG* gene was in the reverse orientation to *LUC*. pFHLG, which lacks the inducible promoter, was derived from pFHLTG by digestion with *Apa*I and *Sal*I, blunt-ending with Klenow to generate a 10.4 kb fragment, which was gel-purified and re-ligated.

2.3. Southern analysis

Genomic DNA was extracted from *Leishmania* using a method adapted from Bellofatto and Cross [14]. *Leishmania* genomic DNA was digested with restriction enzymes and separated by 1% agarose gel electrophoresis, stained with ethidium bromide, and transferred to nylon membranes as described previously [7]. Filters were hybridized with [α-^{32}P]dCTP-labeled probes prepared from gel-isolated DNA fragments using the High Prime labeling system (Amersham Life Science). The *LUC* probe was derived from a 1.4 kb fragment generated from *Cla*I and *Hin*dIII digestion of pTBUC [8]. The *HYG* probe was obtained from a 1 kb fragment resulting from *Bam*HI + *Spe*I digestion of pX63hyg [10]. Hybridization was carried out for 3 h at 65 °C in Rapid-hyb solution (Amersham Life Science). The filters were washed in 2 × SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS for 5 min at room temperature followed by two washes in 0.1 × SSC, 0.1% SDS for 20 min at 65 °C.

2.4. Transfection

Methods for cell electroporation and cell plating have been described previously [15]. Briefly, 4×10^7 promastigotes of LSB-51.1 in 0.4 ml electroporation buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM NaH_2PO_4, 6 mM glucose, pH 7.4) were transfected with 5 μg of DNA by electroporation at 480 V, 500 μF, 13 Ω (BTX Electro Cell Manipulator® 600). Cells were cultured for ~24 h in AM medium before spreading on plates containing 0.7% Seaplaque GTG agarose (FMC Bioproducts) in AM media with 20 μg/ml hygromycin to select single colonies.

2.5. Luciferase assay

Recombinant *Leishmania* cells (10^5) were lysed and assayed, in duplicate, for luciferase activity using a Monolight luminometer (Analytic Luminescence Laboratory) following manufacturer's instruction. The luciferase activities for all samples were measured for 10 s. Experiments were repeated several times and the data presented are representative.

3. Results

3.1. Separation of the selectable marker and reporter genes

Construct pFHLTG was designed to express the selectable marker (*HYG*) from an endogenous promoter and the reporter gene (*LUC*) from an inducible P_{RRNA} on the opposite DNA strand (Fig. 1). TETR-ex-

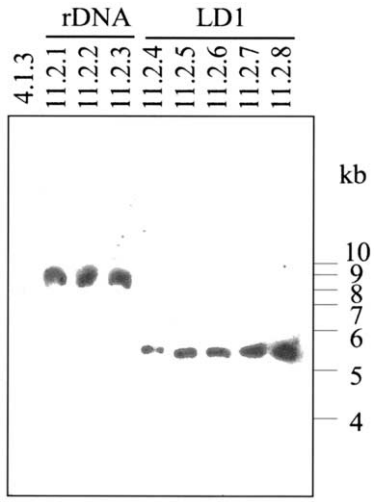

Fig. 2. Integration of the luciferase gene into the *rDNA* and LD1 loci. *Spe*I-digested genomic DNA (1 μg) from cell lines resulting from transfection with pFHLTG (11.2.1–11.2.8), as well as the parental cell line (4.1.3), was hybridized with a 1.4 kb *LUC* probe. The 8.7 kb band is indicative of integration into the *rDNA* locus (Chr27), while the 5.6 kb is indicative of integration into the LD1 locus (Chr35). Molecular size markers (Gibco/BRL) are shown to the right.

pressing cell line 4.1.3 [8] was originally derived from LSB-51.1 cells in which an additional copy of *ORFF* and *BT1* from the LD1 locus is present in the *rDNA* locus [7]. Construct pFHLTG with *TetO* at 2 nt upstream of TIS was targeted into either the *rDNA* or LD1 loci, where the endogenous genes are normally transcribed by pol I and pol II, respectively [7]. Linearized plasmid DNA was transfected into the 4.1.3 cell line that expresses *TETR* from the tubulin locus [8], and hygromycin-resistant cells were selected in the absence of tetracycline. Genomic Southern analyses of eight resultant recombinant cell lines with the *LUC* probe (Fig. 2) showed the 8.7 kb *Spe*I fragment in three clones (11.2.1–11.2.3), diagnostic of integration into the *rDNA* locus on Chr27, while the other five recombinant cell lines (11.2.4–11.2.8) showed hybridization to the 5.6 kb fragment indicative of integration into the LD1 locus on Chr35.

In all cell lines, addition of tetracycline to 10 μg/ml induced luciferase activity by three orders of magnitude (Fig. 3). In the presence of tetracycline, the three recombinant cell lines with integration into the *rDNA* locus (11.2.1–11.2.3) showed more than 10-fold higher luciferase activities than the five clones with integration into the LD1 locus (11.2.4–11.2.8). The similar relative differences in luciferase activity were also seen in the absence of tetracycline, with the five recombinant cell lines with integration in the LD1 locus having a level of expression close to machine background.

3.2. Kinetics of regulated gene expression

Luciferase expression was rapidly induced upon addition of tetracycline, but took several days to return to background level after its removal (Fig. 4A). The time course of response to tetracycline induction was similar in recombinant cell lines from the *rDNA* locus (11.2.2) and LD1 locus (11.2.5), except that the uninduced and induced levels were higher in the former than the latter. After addition of 10 μg/ml of tetracycline, luciferase activity increased rapidly, becoming elevated by ~10–50-fold within 2 h and ~30–100-fold within 5 h after addition, with full induction by 24 h (Fig. 4A). After 48 h, tetracycline was washed away and cells were maintained in antibiotic-free medium. In contrast to the rapid induction of luciferase expression, luciferase activity decreased only 3–4-fold within 4 h, ~10-fold after 24 h, then slowly declined until it reached background level after about 1 week.

The level of luciferase activity was dependent on tetracycline concentration for both cell lines (Fig. 4B). Concentrations above 0.1 μg/ml resulted in a plateau of maximal induction, while 0.001 μg/ml resulted in little or no induction. A concentration of 0.01 μg/ml resulted in an intermediate level of activity. These results showed that *LUC* gene expression could be modulated by adjusting the concentration of tetracycline.

3.3. Background luciferase activity in the absence of promoter

Constructs pFHLG (see Fig. 1) generated cell lines with a promoter-less *LUC* gene adjacent to the *BT1* gene in the *rDNA* locus or LD1 locus, but on the DNA strand opposite to that of the endogenous *rDNA* (and *BT1*) genes. The selectable marker *HYG* gene is on the

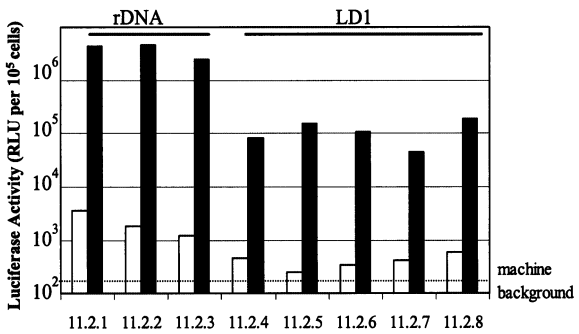

Fig. 3. Luciferase expression from an inducible promoter. Luciferase activity in lysates of 10^5 cells was measured after growth of cell lines resulting from transfection with pFHLTG (11.2.1–11.2.8) in the presence (solid bars) and absence (open bars) of tetracycline. The chromosomal location of the integration event is indicated at the top. The level of luciferase activity due to machine background is indicated by the dotted line. Luciferase activities are given as average relative light units (RLU) from duplicate lysates.

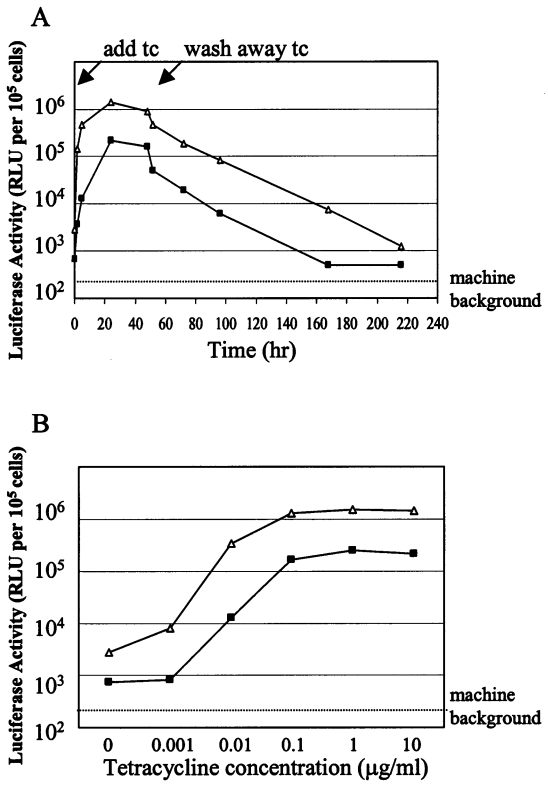

Fig. 4. Response of luciferase expression to tetracycline. (A) Kinetics (tc) of luciferase expression in cells with the regulated reporter gene in the *rDNA* (11.2.2, open triangles) or LD1 (11.2.5, closed squares) loci, after addition of 10 μg/ml tetracycline (0 h) and subsequent washing of the cells and transfer to tetracycline-free medium (48 h). (B) Luciferase activity measured after exposure to different concentration of tetracycline for 24 h. The level of luciferase activity due to machine background is indicated by the dotted line. Luciferase activities are given as average RLU from duplicate lysates.

same strand as the *rDNA* genes or LD1 genes, and is driven by the endogenous promoter. Cell lines derived from pGUH1 contain a promoter-less *LUC* gene on the DNA strand opposite the *rDNA* genes, upstream of a *HYG* gene carrying its own P_{RRNA} (Fig. 1). The expected integration of these constructs was confirmed by Southern analysis (Fig. 5). *Xho*I-digested genomic DNA from recombinant cell lines 8.1.1–8.1.3 hybridized with the *HYG* probe revealed the 6 kb fragment expected from integration of pGUH1 into the *rDNA* locus (Fig. 5A). Hybridization of *Spe*I-digested genomic DNA from cell lines 11.6.1 and 11.6.3 with the *LUC* probe revealed the 8.4 and 5.3 kb fragments predicted from the integration of pFHLG into the *rDNA* and LD1 loci, respectively (Fig. 5B).

Surprisingly, the luciferase activities obtained from these cell lines were quite different, although the *LUC* gene was in a similar position relative to the *BT1* gene in all three cases (i.e. immediately upstream and on the opposite DNA strand). As shown in Fig. 6, the luciferase activities of cell lines

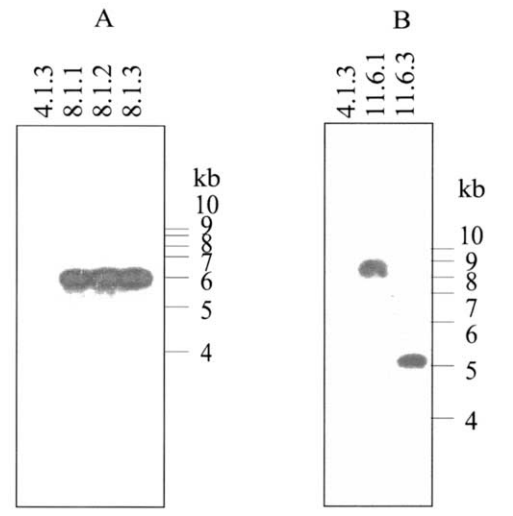

Fig. 5. Integration of the luciferase gene into the *rDNA* or LD1 locus. (A) *Xho*I-digested genomic DNA from cell lines generated from stable transfection with pGUH1 (8.1.1–8.1.3), as well as the parental cell line (4.1.3) was hybridized to a *HYG*-specific probe. (B) *Spe*I-digested genomic DNA of cell lines (11.6.1 and 11.6.3) derived from transfection of 4.1.3 with pFHLG was hybridized to a *LUC* probe. Molecular size markers (Gibco/BRL) are shown on the right.

(8.1.1–8.1.3) derived from construct pGUH1 were 3–4 orders of magnitude higher than those (11.6.1 and 11.6.3) derived from construct pFHLG, which were close to machine background. Thus, it appears that the *LUC* gene is not actively transcribed on the strand opposite to endogenous transcription in the absence of promoter sequence. However, the *LUC* gene is actively expressed in cell lines 8.1.1–8.1.3 which contains exogeneous P_{RRNA} (expressing the *HYG* gene) on the same strand, but downstream of, the *LUC* gene.

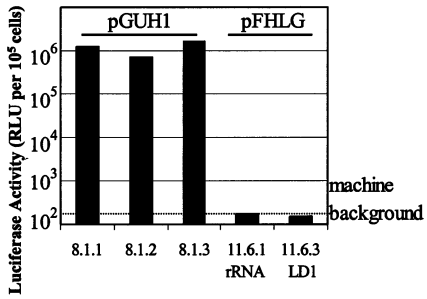

Fig. 6. Luciferase activity on the DNA strand opposite to the *rDNA* genes. Luciferase activity in lysates of 10^5 cells was measured in cell lines containing constructs pGUH1 (8.1.1–8.1.3) and pFHLG (11.6.1 and 11.6.3) integrated into the *rDNA* locus and LD1 locus. The level of luciferase activity due to machine background is indicated by the dotted line. Luciferase activities are given as average RLU from duplicate lysates.

4. Discussion

In this study, we describe an inducible promoter system that allows expression of protein-coding genes to be regulated by a tetracycline-responsive *rRNA* promoter in *L. donovani*. This second-generation system showed tetracycline-mediated regulation of luciferase expression by over three orders of magnitude, with background levels (in the absence of tetracycline) near machine background. The luciferase activity could be modulated within this range by using different concentrations of tetracycline (between 0.1 and 0.001 μg/ml). Activation of the inducible promoter by tetracycline appears to be relatively rapid (several hours), but the decline of luciferase activity was slower (several days), possibly reflecting the stability of luciferase mRNA and protein, rather than promoter activity.

The induced and uninduced levels of luciferase expression were affected by the genomic location of the inducible promoter. The background (uninduced) luciferase activity of cell lines where the pFHLTG construct was integrated into the LD1 locus on Chr35 was only 2–3-fold higher than machine background, but was 10-fold higher than machine background in cell lines with integration into the *rDNA* locus on Chr27. Likewise, the induced levels were over 10-fold higher in the latter cell lines compared to the former. Since the same promoter construct was used in both cases, and the parent cell lines were identical, it appears that the *rDNA* locus permits higher expression levels from the exogenous inducible *rRNA* promoter. This may reflect differences in chromatin structure, nuclear compartmentalization, transcription status (i.e. pol II versus pol I) or some combination thereof of these two loci.

The increased dynamic range of regulated gene expression of this system over the first-generation inducible system [8] is largely due to the lower background in the absence of tetracycline. The first-generation system utilized the same inducible pol I promoter [8] for both the selectable marker (*BLE*) and reporter (*LUC*), since their coding sequences were fused. Thus, recombinant cell lines containing this construct had to be selected in the induced state (i.e. in the presence of tetracycline). This may have resulted in selection of cells with higher background levels of expression of the DNA strand containing the inducible promoter and *BLE-LUC* gene (i.e. the strand opposite the endogenous *rDNA* genes). The first-generation system used phleomycin selection, rather than hygromycin, which was used in the second-generation system. The *BLE* gene product acts by binding phleomycin and preventing its interaction with parasite DNA [16], and probably requires higher intracellular expression levels than the *HYG* gene product, which acts enzymatically to inactivate hygromycin. This may have been exacerbated by the use of a *BLE-LUC* fusion protein, which could have decreased phleomycin-binding capacity, and *DHFR-TS* flanking sequences (compared to *DST* flanking sequences for the second-generation system) which may have adversely affected reporter *BLE-LUC* mRNA stability and/or translation.

Transcription of sequences upstream of the exogenous pol I promoter in the *rDNA* locus occurs when the promoter is present, but not when it is absent. The DNA strand opposite the endogenous *rDNA* genes is normally not transcribed at an appreciable level, as indicated by the lack of luciferase activity in cell lines that contain a promoter-less *LUC* gene integrated in the *rDNA* locus (pFHLG, Fig. 6). However, in cell lines (pGUH1, Fig. 6) that contain an active exogenous pol I promoter downstream of, and on the same strand as, the *LUC* gene, substantial luciferase activity is detected. Thus, sequences both upstream and downstream of the promoter on this DNA strand are transcribed in this cell line. While it is possible that the result may be an artifact of selection for cells that transcribed this DNA strand in order to be hygromycin-resistant, an intriguing possibility is that the active promoter may have resulted in alteration of chromatin structure nearby. Such an effect could include differences in modified DNA bases such as base J [17] or proteins such as histone and HMG protein acetylation or phosphorylation [18,19]. Insight into these processes would be valuable to the understanding of control of gene expression in *Leishmania*.

Overall, we developed an improved tetracycline-responsive inducible system so that control of expression from a pol I promoter allows regulation by three orders of magnitude. The ability to control promoter activity will be useful for studying gene function in this pathogen. This system may be useful for controlling the expression of genes at defined lifecycle stages, especially for essential genes and when gene replacements are lethal. Applications of this inducible system for studying gene function in *Leishmania* are currently in progress.

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