In vitro transcription of mutated *Leishmania tarentolae* spliced leader RNA genes approximates in vivo patterns

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Received 7 July 2000; accepted 5 September 2000

Abstract

To elucidate the process of transcription in the kinetoplastids and to aid in the purification of transcription factors, we have developed a transcriptionally-competent nuclear extract from *Leishmania tarentolae* for the study of the spliced leader (SL) RNA gene. The extract was competent to transcribe a tagged SL RNA gene. The in vitro SL RNA transcripts initiated accurately and their synthesis was dependent on the presence of the promoter defined in vivo. The nuclear extract was then challenged rigorously using an exhaustive set of mutated SL RNA gene templates previously tested for transcriptional activity in vivo. Mutation of four nucleotides (CCGG) at positions −34 to −31 had a detrimental effect on transcription in vitro; the CC dinucleotide overlaps one element necessary in vivo. Similarly, four nucleotides (TGAC; positions −67 to −64) important for transcription in vitro overlapped the other core promoter element defined in vivo, but were generally not effective as point mutations. The promoter-binding ability of the transcriptionally-competent extract for the −60 region mutations mirrored the in vitro transcription pattern. Although it does not reflect precisely the in vivo results, this in vitro system provides us with an important tool for monitoring the purification of potential transcription factors, as well as the basis for future reconstitution experiments. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Kinetoplastid; Mini-exon; snRNA; Trans-splicing; Trypanosome

1. Introduction

Elucidation of the sites for and mechanisms of RNA polymerase (RNA pol) II transcription initiation in the kinetoplastid protozoa has been arduous. The inability to identify an indisputable RNA pol II promoter and the occurrence of polycistronic transcription of nuclear-encoded pre-mRNAs [1,2] have contributed to the complications. Of all the promoters identified in the
trypanosomes, the variant surface glycoprotein and procyclin genes of *Trypanosoma brucei* are the best studied for protein-encoding genes [3]. Contrasting the typical RNA pol II-directed transcription of housekeeping genes, these promoters were found to direct transcription by an RNA pol I-like activity [4]. At least three possible RNA pol II promoters have been proposed in kinetoplastids: one within the 70-kDa heat shock protein gene array of *T. brucei* [5]; one for the glutamic acid/alanine-rich protein gene in *T. congolense* [6] (NB, contradictory results have been obtained for this promoter [7]); and one for the spliced leader (SL) RNA gene promoter [8–10]. In contrast to the first two examples, where the final gene products are proteins, the product of the SL RNA (a.k.a. mini-exon) gene is a small, discrete RNA molecule. In *Leishmania* the SL RNA is 96 nt in length. Via the trans-splicing reaction, a process central to nuclear RNA maturation in kinetoplastids, the 39-nt SL becomes the 5′ end of all nuclear mRNAs [11].

The polymerase that drives the transcription of the SL RNA gene is a matter of debate [12]. The SL RNA gene promoter is proposed to direct transcription by RNA pol II in *T. brucei* [8], *T. cruzi* [10] and *L. tarentolae* [9]. However, in *T. brucei* [13,14] and *Crithidia fasciculata* [15] RNA pol III is implicated for transcription of the SL RNA gene. A polymerase activity distinct from classical RNA pol II and RNA pol III has also been suggested [16,17]. Further characterization of the SL RNA gene promoter architecture and the interacting transcription factors will facilitate understanding of the mechanism of SL RNA gene transcription initiation.

We have used stable transfection to map the *L. tarentolae* SL RNA gene promoter to the level of individual nucleotides [18], making this the best defined potential RNA pol II promoter in kinetoplastids. The core nucleotides of two promoter elements are GACN5G at nucleotide positions −67 to −58 and GN3CCC at nucleotide positions −39 to −33 upstream of the SL RNA gene. Additionally, we have shown that the nucleotides essential for in vivo transcription within the GACN5G element are also necessary for a specific protein-DNA interaction [18]. In contrast to *Lep- tomonas* [19] and *T. brucei* [16], the *L. tarentolae* promoter does not contain essential nucleotides immediately upstream of the transcription start site [18].

A useful complement to in vivo studies is a transcriptionally-competent extract that can mirror in vivo fidelity. However, in vitro accuracy may be affected by the enrichment of the transcription machinery in nuclear extracts, which could push the system in the direction of transcription initiation [20]. Kinetoplastid-based in vitro transcription systems have been reported for *T. brucei* [16,21] and *Leptomonas seymouri* [22]. Our in vitro transcription system in *L. tarentolae* recognized the in vivo termination signal for the SL RNA gene, providing important insight into the 3′-end formation pathway [23]. Here we report rigorous testing of this extract for transcriptional initiation in comparison with our extensive in vivo analysis [18]. The interaction of components from the transcriptionally-competent extract with −60 region promoter mutations was also addressed using the electrophoretic mobility shift assay (EMSA).

2. Methods

2.1. Plasmids

SL RNA gene promoter mutation plasmids have been described previously: the intron-tag 10-bp scan mutations [9], the intron-tag dinucleotide and point mutations [18], and the exon-tag −67/−58 mutation [24].

2.2. Preparation of *L. tarentolae* transcriptionally-competent nuclear extract

A one liter culture of *L. tarentolae* cells was grown in BHI medium to a density of 5 × 10⁷ cells ml⁻¹ [18]. Cells were harvested at 4°C and cell pellets were washed twice with 50 ml of ice-cold 1X PBS. All the subsequent steps were performed on ice. The rinsed cell pellets were resuspended in buffer A (10 mM HEPES-KOH (pH 7.9), 20 mM potassium L-glutamate, 3 mM MgCl₂, 150 mM sucrose, 1 mM DTT, 1 mM PMSF, 0.7 μg ml⁻¹
pepstatin, 2 μg ml⁻¹ leupeptin) for 15 min. The cells were centrifuged in a Beckman JA-20 rotor at 3000 rpm for 10 min at 4°C and the cell pellet resuspended in 1.5 times packed cell volume of buffer A for 5 min. The swollen cells were disrupted by continuous plunging in a chilled glass dounce (type A pestle) for 10 min. Cell lysates were centrifuged for 10 min at 3000 rpm in a Beckman JA-20 rotor at 4°C. The nuclear pellets were resuspended in an equal packed cell volume of buffer A and 3 M potassium L-glutamate was added in a dropwise fashion to achieve the final concentration of 0.4 M. This resuspension was ultracentrifuged using Beckman SW 55 Ti rotor at 40000 rpm for 1 h at 4°C. The supernatant was dialyzed overnight in dialysis buffer (10 mM HEPES-KOH (pH 7.9), 100 mM potassium L-glutamate, 3 mM MgCl₂, 150 mM sucrose, 0.1 mM EDTA, 1 mM DTT and 1 mM PMSF). Extracts were stored at −80°C where they were stable for more than 1 year.

2.3. In vitro transcription analysis

The transcription reactions were performed by incubating approximately 1 μg of ‘Qiaprep’-prepared test plasmid DNA (Qiagen) with 10 μl of nuclear extract prepared as described above, 20 mM potassium L-glutamate, 10 mM HEPES (pH 7.9), 2.5 mM MgCl₂, 1 mM DTT, 10 μg ml⁻¹ leupeptin, 100 μg ml⁻¹ pepstatin, 40 μ of porcine RNase inhibitor (Pharcmaica), 2.5% PEG, 1.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.5 mM UTP, 20 mM phosphocreatine, and 12 μg phosphocreatine kinase, for 10 min at 28°C. In reactions using mutated templates, 0.25 μg of an exon tagged (tSL; [24]) plasmid was included as a positive control for extract activity and an internal normalizer.

2.4. RNA analysis

RNA from the in vitro reactions was extracted using TriZOL reagent (Gibco-BRL). Transcripts were analyzed by primer extension as described previously [24]. Oligonucleotide probes were prepared by 5'-end labeling with [γ⁻³²P]dATP and T4 polynucleotide kinase. Oligonucleotide M838 Jr was used when the DNA template contained the intron-tag (IT; [9]); the oligonucleotide 28/39-tag was used to perform primer extension when the DNA template contained tSL. Quantitation of bands was performed with a PhosphorImager (Molecular Dynamics).

2.5. Electrophoretic mobility shift assay

A set of labeled, double stranded binding substrates was generated using oligonucleotides from the WT–70–46e series and DECA–70–46e; binding reactions were performed as described [18] and electrophoresed (100 V, 3.5 h) in a 4% native polyacrylamide gel in 0.5X TBE buffer. Quantitation and normalization of band intensity was performed as described previously [18] using a PhosphorImager.

2.6. Oligonucleotides

The following oligonucleotides were used in this study: 28/39-tag (5'-ACTTC CTCGA GGCTG AA) and M838 Jr (5'-AGCCT TGTGG GCCAG TG). The WT–70–46e series and complementary DECA–70–46e have been described elsewhere [18].

3. Results and discussion

3.1. L. tarentolae nuclear extracts accurately initiate transcription of SL RNA and tRNA gene templates

Previously, we have studied the cis-acting (DNA) determinants of transcription for a plasmid-borne, tagged L. tarentolae SL RNA gene in stable transfectants [9,18,23]. We have identified two specific transcription elements in vivo, either of which will reduce or eliminate generation of the tagged SL RNA when mutated [18]. Since in vitro transcription systems have been developed for other kinetoplastid protozoa [16,21,22], we wished to compare the fidelity of transcriptionally-competent L. tarentolae extract first with in vivo transcription initiation data, and second for the content of trans-acting protein factors identified.
from transcriptionally-incompetent extracts [18]. The ability of *L. tarentolae* nuclear extract to effect accurate transcription of the SL RNA gene was tested using a plasmid template (tSL) that was functional in transfection studies [9,23]. Transcripts generated during the in vitro transcription reaction were identified by reverse transcriptase primer extension with oligonucleotide 28/39-tag, which hybridizes to tSL.

Two SL RNA gene templates were tested initially in the nuclear extract: a tSL plasmid, which contained a wild-type promoter and the exon-tag, and a tSL ‘promoter knock-out’ plasmid that contained a mutation of nucleotides −67 to −58 that inactivates specific transcription of the SL RNA in vivo [24]. After 10 min incubation of plasmids with the *L. tarentolae* nuclear extract, transcripts that corresponded to SL RNA could be detected from the WT promoter template (tSL) but not from the inactive promoter template (−67/−58 + tSL) by primer extension (Fig. 1). Parallel electrophoresis of a dideoxy DNA sequencing reaction of the plasmid template revealed that the primer extension products extended to the in vivo transcription start point (+1). This result is consistent with precise promoter-driven transcription initiation by the nuclear extract, and with a lack of modification of in vitro-generated transcripts [16,22] to produce the cap 4 structure that typically blocks progress of the reverse transcriptase at nucleotide +5 (see also lanes WT (in vivo) and WT in Fig. 2). In all subsequent transcription reactions promoter mutation templates contained a 40-nt insertion tag within the intron and were assayed in at least three separate experiments in the presence of the tSL template as an internal positive control for extract activity and mutation transcriptional efficiency.

### 3.2. The in vitro transcription assay identifies the two upstream blocks that regulate SL RNA gene transcription in vivo

Having established an in vitro transcription assay for the SL RNA gene that was faithful for initiation and termination [23], we tested our existing 10-bp linker-scan mutation series (Fig. 2A) to determine whether the in vitro assay reproduced the results obtained in vivo. RNAs resulting from incubation of these constructs in transcriptionally competent *L. tarentolae* nuclear extracts were detected by primer extension with two oligonucleotides, one that detects the 140-nt intron-tagged (IT) transcript and the other that detects the control 96-nt exon-tagged transcript (Fig. 2B).

The in vitro extract was unable to transcribe the intron-tagged gene that contained the −67/−58 mutation, identical to the in vivo situation. Mutation of nucleotides −40/−31, which eliminated transcription in vivo, showed a reduced level of transcription (24% of WT) from the SL RNA gene. The remaining nine intron-tagged templates yielded the expected extension products. Thus, with regard to the gross configuration of the promoter, the in vitro extract recognized faithfully the −67/−58 upstream element defined in vivo and implicated the −40/−31 element by significantly reduced efficiency of transcription. The mutation of positions −1/9 yielded a slightly reduced level of transcript in vitro, consistent with

![Fig. 1](image-url)
Fig. 2. Two upstream promoter elements are implicated in SL RNA gene transcription in vitro. (A) The positions of linker-scan mutations in the region −80 to +19 of an intron-tagged SL RNA gene [9]. Nucleotides not altered by the scan sequence, CCTCGAGGAA, are indicated by dots. The arrow represents the transcription start site. (B) Primer extension analysis of RNA transcribed in vitro from linker-scan mutated SL RNA gene plasmids. Total RNA from each in vitro transcription reaction containing both the plasmid with linker-scan mutation and a plasmid with a wild-type promoter/exon-tagged SL RNA gene (tSL) was used in a primer extension reaction with 5’-end labeled oligonucleotides M838 Jr and 28:39-tag. IT represents the intron-tagged SL RNA from linker-scan mutation plasmids and tSL represents the transcript from the exon-tagged SL RNA gene (with wild type promoter) plasmid as an internal control for the in vitro transcription reaction.

the 2–3 fold reduced level of transcription in vivo [9]. A minor band two nucleotides below the expected product may represent an alternate start site as observed in other kinetoplastids [16,19] or possible cap modification in vitro. Such activity has been observed with similar extracts from T. brucei on SL ribonucleoprotein particle substrates, however the activity was not observed for purified RNA templates [25]. Consistent with our results in vivo, mutation of positions −11/−2 had no significant effect on transcription initiation in vitro in L. tarentolae. This observation contrasts with both Leptomonas [19] and T. brucei [16], where mutation of these positions resulted in altered transcription start site usage in vitro and in vivo. Such discrepancies may reflect evolutionary differences in the SL RNA gene promoter architecture among the kinetoplastids (reviewed in [12]).

3.3. CCGG at −34/−31 modulates SL RNA gene transcription in vitro

Additional block, dinucleotide and point mutations (Fig. 3A) in the −41/−31 region were tested in the in vitro assay, following our approach for mapping the promoter elements in vivo. We had tested previously equivalent regions from related kinetoplastids (Cf, Lm, Lt2) and particular motifs (−35 CCC −33) as well as a dinucleotide transversion series for transcription efficiency in vivo [19]. In vitro transcripts from promoter-mutation plasmids (IT) and the control WT-promoter plasmid (tSL) detected by primer extension are shown in Fig. 3B.
The original linker-scan mutation −40/−31 showed reduced but detectable levels of transcription in vitro, whereas the shorter mutations within this region had greater effects. This could be due to the precise nature of the mutations. The reduction in transcription seen in vivo for the replacement mutations from *Crithidia*, *L. major* and the second *L. tarentolae* SL locus were not detected by the in vitro system. Likewise, significant single nucleotide changes in vivo such as those at position −39 or −35 had no effect on in vitro activity. Mutation of nucleotides −38/−33, −35/−33 and −34/−33 resulted in significantly reduced transcript levels (≤20% of WT) relative to the tSL transcript, consistent with the in vivo results. Mutation of −32/−31 reduced transcription in vitro but not in vivo. Intermediate effects (30–55% of WT) were observed for mutations −36/−35 and −40/−39. Thus, of the core GN4CC element defined in vivo, the 3′ CC and the downstream adjacent GG dinucleotide had the strongest effects on efficient in vitro transcription.

### 3.4. Dinucleotide mutation of TGAC within the −67−58 region most effectively inhibits SL RNA gene transcription in vitro

Block mutation of the −67/−58 region completely inhibited transcription in vivo and protein binding in vitro [18], and transcription in vitro (Fig. 2B). Thus we subjected the detailed mutagenesis series of this region (Fig. 4A) to the in vitro transcription assay. The effects of dinucleotide- (Fig. 4B) and single-nucleotide (Fig. 4C) transversion mutations were assayed by primer extension as in the previous sections. In general the in vitro results for the dinucleotide mutations agreed with the in vivo data: mutation of nucleotides −67/−58, −67/−66 and −65/−64 inhibited transcription in vivo and in vitro (transcript level ≤20% of WT). Mutation of nucleotides −69/−68, −57/−56 and −53/−52 had no effect in either system. In contrast, mutation of positions −59/−58, −67, −66, −65 and −58, which impaired SL RNA gene transcription in vivo, had little or no intermediate effects on SL RNA gene transcription in vitro. Although mutation of the TG (−67/−66) and AC (−65/−64) dinucleotides was detrimental, only point mutation of position −64 had a significant effect on transcription in vitro. Thus, of the GACN4G core nucleotides defined in vivo, dinucleotide combinations were required to adversely affect transcription in vitro with the exception of position −64.

For both upstream elements the nucleotides...
required for efficient transcription in vitro overlapped the nucleotide blocks defined in vivo. The lack of a perfect correlation between the in vivo and in vitro results is not surprising. Similar inconsistencies have been observed in 10-bp linker-scan mutagenesis of the \textit{T. brucei} SL RNA gene upstream region [16]. A possible explanation for these results is the nature of the extracts. Some specificity may be lost in the presence of high concentrations of protein, similar to the proposed behavior of transcriptional activators [20]. Alternatively the extract may be deficient for some components that provide specificity in the in vivo transcription initiation complex. Another consideration is the topology of the plasmid templates. Given that transcription of some episomal genes may be compromised in vivo [26,27], superhelical, bacterially derived, plasmid DNA could adversely affect transcriptional fidelity in vitro. Likewise the presumptive absence of nucleosome participation in the in vitro system may affect the outcome of the experiment.

3.5. \textit{Protein-DNA interaction profile using the transcriptionally-competent extract correlates with the in vitro transcription profile}

There is a correlation between the nucleotides necessary for protein-DNA interaction in vitro and those necessary for SL RNA gene transcription in vivo [18]. However the \textit{L. tarentolae} nuclear extract used in the gel shift analysis was prepared differently from the transcriptionally-competent \textit{L. tarentolae} nuclear extract described here. The major distinction lies in the constituents of the solutions: the transcriptionally-competent extract contained a sucrose-based buffer with K-glutamate; the gel-shift extract contained glycerol and KCl and was not capable of transcribing DNA. Because a future goal of these experiments is to purify the transcription factor(s) for the SL RNA gene, we determined whether the DNA-binding activity was identical in both extracts. This comparison is important in light of the observation that some of the core nucleotides essential for SL RNA gene promoter activity in vivo

Fig. 4. Mutation of the nucleotides TGAC at positions -65/ -64 has the most significant impact on in vitro transcription of SL RNA gene. (A) Summary of mutations constructed within positions -69 to -48. Dots represent unchanged nucleotides. Bold uppercase letters represent mutations that reduced the transcript level to less than 20% of WT. (B) Primer extension analysis of total RNA from in vitro transcription reactions that contained plasmids with 2-bp transversion mutations within positions -69 to -48 and tSL. IT represents tagged transcripts and tSL represents wild-type exon tagged SL RNA gene construct. (C) Analysis of total RNA from in vitro transcription reactions that contained plasmids with single-bp transversion mutations and tSL. Primer extension was performed with 5'-end labeled oligonucleotides M838 Jr and 28/39-tag.
appear dispensible for in vitro transcription (Fig. 4).

Because dinucleotide mutations were necessary to observe detrimental effects in the in vitro transcription assay, we tested the transcriptionally-competent extracts for the ability to bind to 2-bp mutations within the −67/−48 region in which promoter/protein-binding interactions occur (Fig. 5). Loss of the specific gel shift [18] was observed when the extract was incubated with substrates mutated at positions −67/−58 and −65/−64. Reduction of the specific gel shift was observed using mutated substrates −67/−66 (reduced to 40%), −59/−58 (reduced to 56%) and −49/−48 (reduced to 26%). The only point mutation, nucleotide −64, to eliminate protein binding mirrored the in vitro transcription result (Fig. 4C). Thus, although the DNA-binding profile of the transcriptionally-competent extract matched the in vitro transcription profile, it was not consistent with data obtained using our transcriptionally-inactive extracts [18]. Generation of an accurate in vitro transcription extract may rely on diluting or balancing specific components to reflect the environment of the promoter in nucleo.

The presence of a promoter-binding activity in the in vitro transcription extracts provided a further justification for the biochemical purification of the component proteins [28]. Purification of the *Leishmania* SL RNA gene transcription factor will lead to the identification of components within the complex responsible for SL RNA gene transcription. The in vitro transcription assay is a major advance toward development of reconstituted transcription assays to test the function of purified transcription factors. Furthermore, the in vitro transcription assay will provide an additional route to obtain a definitive answer regarding to the identity of the RNA polymerase transcribing the SL RNA gene in *L. tarentolae* via the immunological detection [29] of template-bound polymerase [30] in an in vitro transcription reaction.

**Acknowledgements**

We thank Arthur Günzl for advice on making in vitro transcription extracts. This work was supported by NIH grant AI34536. M.C.Y. and N.R.S. received support from the Microbial Pathogenesis Training Grant (NIH T32-AI07323).

![Fig. 5](image)

*Fig. 5. Protein(s) in the transcriptionally-competent nuclear extract bind to the −67/−58 promoter element. Radiolabeled substrates containing 2-bp transversion mutations from nucleotide positions −69 to −48, and point mutations from −67 to −64 and −58 and −59. PDC = protein-DNA complex, FP = free probe. The two WT substrates contain wild-type promoter sequences from −69 to −48 (WT−69/−48e) or −69 to −36 (WT−69/−36e).*
References


