Alpha-amanitin-resistant transcription units in trypanosomes: a comparison of promoter sequences for a VSG gene expression site and for the ribosomal RNA genes

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ABSTRACT

Transcription of the predominant surface antigen genes in Trypanosoma brucei is unusual in its resistance to the RNA polymerase inhibitor α-amanitin, a property typical for rDNA transcription in eukaryotes. Transcription of most other protein-coding genes in trypanosomes is sensitive to α-amanitin. To investigate whether RNA polymerase I, the polymerase that transcribes rRNA genes, can give rise to functional mRNAs in trypanosomes, we have fused the putative promoter of the T. brucei rRNA genes to the chloramphenicol acetyl transferase (CAT) gene and determined CAT activity after transient expression of chimeric constructs in procyclic trypanosomes. We show here that the rRNA promoter yields the same high CAT activity as the promoters for the two predominant surface antigen genes of trypanosomes, the Variant-specific Surface Glycoprotein (VSG) gene of bloodstream trypanosomes and the procyclin gene of insect-form trypanosomes, both of which are also transcribed by an α-amanitin-insensitive RNA polymerase. RNA polymerase I of trypanosomes seems therefore able to synthesize pre-mRNAs that are effectively processed into translatable mRNAs. Dissection of the promoter segments showed the minimal elements for a VSG gene expression site promoter to be confined to a segment of −60 to +77 bp, overlapping the most 5′ putative transcription start sites as determined in vivo by RNase protection experiments1. For the ribosomal promoter region a segment of −258 to +200 bp relative to the putative transcription start site was sufficient for maximal CAT activity. There is a precise requirement for specific nucleotides at the rRNA transcription start site. We detect no homology between the sequences required for promoter function of the three α-amanitin-resistant transcription units, rRNA, VSG and procyclin (parp) genes. This suggests that the sequence-specific recognition of these promoters either occurs by common factors detecting sequence homologies that escape us, or by separate factors that bind to different DNA sequences but interact with a common α-amanitin-resistant RNA polymerase.

INTRODUCTION

Different classes of RNA polymerases can be distinguished in eukaryotic cells using biochemical criteria such as chromatographic separation and differential sensitivity to the bicyclic octapeptide, α-amanitin. RNA polymerase I (pol I) is resistant to high concentrations of α-amanitin and mediates the transcription of ribosomal RNA genes (rDNA), while RNA polymerase II (pol II) is highly sensitive to low concentrations of the drug and takes care of the transcription of protein-coding genes. RNA polymerase III (pol III), with an intermediate sensitivity, provides the cell with small RNAs, such as 5S and tRNAs2,3,4.

In African trypanosomes exhibiting antigenic variation, such as T. brucei, most of the protein-coding genes are transcribed by an α-amanitin sensitive RNA polymerase, probably pol II, like in other eukaryotes. However, the transcription of genes for the predominant surface antigen of bloodstream form trypanosomes, the Variant-specific Surface Glycoprotein or VSG, in telomeric expression sites (ESs) is α-amanitin insensitive6. The same holds true for the transcription of the gene for the insect stage counterpart of the bloodstream form VSG, the procyclin gene. The transcription of these surface antigen genes is hardly affected by concentrations of α-amanitin as high as 1 mg per ml, whereas that of most other protein-coding genes such as the α- and β-tubulin genes is half-maximally inhibited at 5 μg per ml9.

What transcription machinery mediates this α-amanitin-insensitive transcription of protein coding genes; the intrinsically resistant pol I, or a specialized resistant pol II-like activity? Support for the latter hypothesis has come from the identification of two genes for the largest subunit of pol II. These genes differ

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by 25 nucleotide substitutions, four of which result in amino acid changes\textsuperscript{10,11}. One of these pol II largest subunit proteins might enable this enzyme to mediate $\alpha$-amanitin-resistant transcription\textsuperscript{5,10}. Support for pol I involvement has come from the description of putative transcription reinitiation sites in VSG gene ESs\textsuperscript{12,13} that show similarity to the putative transcription initiation site for the ribosomal RNA genes\textsuperscript{14}.

Attempts to distinguish between those two hypotheses by direct experiments have failed thusfar. Antibodies discriminating between pol I and pol II do not inhibit run-on RNA synthesis in isolated nuclei (A.W.C.A. Cornelissen, personal communication). Attempts to set up faithful transcription initiation with trypanosome extracts have failed. Fractionation of trypanosome RNA polymerases by fast protein liquid chromatography on Mono-Q and DEAE-Sephadex only yielded the standard three RNA polymerases and no fourth peak of an $\alpha$-amanitin-resistant type II polymerase\textsuperscript{5}.

We have therefore used an indirect approach to get more insight in the nature of the polymerase that transcribes VSG gene ESs. We have determined whether authentic rRNA gene promoters can drive mRNA synthesis in \textit{T. brucei} and whether the minimal promoters of rRNA and VSG gene ESs defined by deletion analysis have any sequence in common.

**MATERIALS AND METHODS**

Trypanosomes

The trypanosomes used belong to strain 427 of \textit{T. brucei brucei}\textsuperscript{15}. Procyclic (culture) form trypanosomes were grown in the semi-defined medium as described\textsuperscript{16}.

Recombinant DNA

The plasmids used in the procyclic transfection experiments were constructed as follows: into the blunted SacI site of the pJF6dSca vector (see Fig. 2), encompassing the parpA splice acceptor region fused to the CAT gene\textsuperscript{1}, blunt-ended promoter fragments were subcloned: (1) the 519 bp AluI fragment (Fig. 5) at the 5' end of the 18S gene in the ribosomal transcription unit of \textit{T. brucei}, overlapping the putative ribosomal promoter region\textsuperscript{14} (Fig. 1 A) in the correct (pRK7(+)) and reverse (pRK7(-)) orientations relative to the CAT gene (Fig. 2); (2) the 2.6 kb Sall fragment from the VSG gene 221 ES, overlapping the promoter for this ES in the correct (pRK8(+)) and reverse (pRK8(-)) orientations\textsuperscript{1} (Fig. 2); (3) the 1166 bp EcoRV fragment, the 748 EcoRV-Sacl, the 597 bp HaeIII, the 90 bp HaeIII-EcoRV and the 137 bp HaeIII fragment from the 221 ES promoter region (depicted in Fig. 3). To obtain 5' deletion mutants of pRK8(+), we subcloned the promoter-parpA splice acceptor-CAT gene segment as a partial EcoRI-BamHI fragment in Bluescript M13(+). The ribosomal promoter 5' deletion constructs were prepared by 5' to 3' unidirectional deletion with Exonuclease III\textsuperscript{17}, and verified by sequencing.

DNA transfection of procyclic trypanosomes by means of electroporation

The method was essentially as described\textsuperscript{1,19,20}. We used in all transfections between 1 and 10 $\mu$g specific plasmid DNA and a constant amount of 50 $\mu$g DNA per 2.5 $\times$ 10\textsuperscript{7} trypanosomes, by electroporation. The method was essentially as described\textsuperscript{1,19,20}.
adding pGEM3 plasmid DNA (Promega). Every clone was tested in at least three independent electroporation experiments. The DNA was prepared by alkaline lysis, and RNA was removed by RNase A digestion followed by phenol-chloroform extraction and ethanol precipitation. The extent of supercoiling in the plasmid DNA did not seem to be a critical factor in the CAT-gene activity as plasmid DNA preparations in which 90% was negatively supercoiled yielded CAT activities similar to those having only 10% supercoiled and mostly open circular DNA. Linearised plasmids, however, yielded very low levels of CAT activity (data not shown). The CAT activity was assayed as described, using 250 μM n-butyryl CoA (Sigma) and 88 μM D-threo[dichloroacetyl-1,2-14C]-chloramphenicol (NEN, DuPont) at a final specific activity of 59.5 mCi per mmole. To stay within the linear range of the CAT assay we incubated 10–20 fold diluted trypanosome transformant extracts for 1 hr at 37°C in these assays. After xylene and two aqueous phase extractions, the xylene fractions were dried, resuspended in ethyl acetate and spotted onto silica plates, which were subsequently developed in 95:5 (v/v) chloroform-methanol. Alternatively, before drying 50 μl of the xylene fractions was mixed with 5 ml of Opti-fluor (Packard) and radioactivity was determined by liquid scintillation counting. CAT activities were correlated with a standard dilution series of E.coli chloramphenicol acetyl transferase (Pharmacia). One unit of this enzyme catalyzes the acetylation of one nanomole of chloramphenicol per minute at 37°C.

RESULTS

Deletion analysis of a VSG gene expression site promoter

The VSG gene 221 ES is controlled by a single promoter 60 kb upstream of the gene. From an analysis of nascent RNA by hybridization and RNase protection we concluded that the transcription of the 221 ES starts in the Scal-SalI fragment (the +1 and +39 start sites; Fig. 1B and 3). The rapid processing of nascent transcripts from VSG gene ESs made it difficult, however, to locate transcription start sites unambiguously. To

Figure 2. Schematic presentation of promoter-parpA splice acceptor-CAT gene constructs used in the transfection of procyclic T.brucei. The detailed description of pJF6 is in ref. 20. This construct contains the parpA promoter (open box) and parpA α gene splice acceptor (dotted box with the AG dinucleotide, indicated by a triangle, at 75 bp downstream of the Scal site and 30 bp upstream of the HindIII site) fused to the CAT gene (hatched box). pJF6Sca, from which the parpA promoter had been deleted, has been described. pPRK(+) and pPRK7(+) contain the VSG gene 221 ES and ribosomal promoter, respectively, and are described in the Materials and Methods. Arrows represent transcription start sites, for the parp promoter24, for the 221 ES promoter7 and for the ribosomal promoter14. Restriction sites: B, BamHI; H, HindIII; K, KpnI; R, EcoRI; S, SalI; Sc, Scal.

Figure 3. Analyses of CAT-gene expression controlled by the VSG gene expression site promoter in transient transfections of procyclic T.brucei. A. A physical map of the promoter region for the VSG gene 221 ES. The hatched box represents the 50 bp repeat array and the two flags mark the putative transcription start sites at +1 and +39 in the 221 ES, as mapped in vivo7. The positions of the restriction sites relative to the +1 start are indicated above the map. Underneath the map the constructs used in the transfection experiments, with their 5' deletion endpoints indicated. The thin line represents promoter region sequences, the dotted box the parpA splice acceptor region and the hatched box the CAT-gene. The corresponding CAT activities for the constructs are in units E.coli CAT (x 100) per 1 x 10^7 trypanosomes. The CAT activity for a particular construct tested in at least three independent transfection experiments varied by at most a factor two in absolute CAT activity, with only 10–20% variability in the relative CAT activities for different constructs within one experiment. The CAT activity for one complete series of constructs is shown. Restriction sites: EV, EcoRV; H, HaeIII; S, SalI; Sc, Scal. B. CAT expression from CAT-gene constructs containing different restriction fragments of the VSG gene 221 ES promoter in transfected T.brucei. One representative experiment is shown and CAT activities are presented as in A. C. Nucleotide sequence of the promoter region for the VSG gene 221 ES. The sequence depicted starts at a Scal site 390 bp upstream of the transcription start site at +1 and extends to the SalI site that ends 126 bp downstream of +1. Shaded sequence represents the area where in 5' deletion analysis the transition occurs from active to inactive promoter sequences. The second 5' end of transcripts in the 221 ES promoter map to position +39. Restriction sites are underlined.
assess the role of the 5' flanking sequences in the transcription initiation region of the VSG gene 221 ES, we have tested deletions in the 221 ES promoter fused to a reporter gene in procyclic (insect form) trypanosomes. Although procyclic trypanosomes do not make VSG mRNA, the VSG gene ES promoter is fully active in this phase of the life cycle, as first shown for endogenous promoters\(^1,24,25\) and subsequently for transfected VSG promoters\(^1,26,27\).

Deletions in the ES promoter were cloned in the basic construct depicted in Figure 2 (pF6dSca). This construct by itself does not contain promoter sequences, but includes all of the bacterial chloramphenicol acetyl transferase (CAT) protein coding sequences and the trans-splicing signals of the parpA gene\(^1,26\). 3' Polyadenylation signals were not present in the construct. Polyadenylation occurs downstream of the CAT-gene in vector sequences and the addition of a polyadenylation signal of the 8-tubulin gene downstream of the CAT gene had only a minor effect on CAT activity (at most a four-fold increase) (data not shown).

5' deletion-constructs containing the VSG gene 221 ES promoter region extending from −2150, stepwise to −66 bp relative to the +1 start and ending at position +126 (Fig. 3 A) all yielded similar CAT activities in procyclic trypanosomes, suggesting that no major regulatory sequences are located immediately upstream of position −66. Further deletion of sequences up to +3 reduced CAT activity approximately 100 fold, however. These results suggest that sequences in the region between −66 to +3, a total of only 69 bp, are important in VSG gene expression in procyclic trypanosomes. This region overlaps with one of the two 5' ends of promoter proximal precursor RNA molecules (at +1) previously mapped to this region (Fig. 3 and ref. 1). The construct containing the region −2480 to −2155, which encompasses part of the 50 bp repeat region (from −2480 to −1320 bp) in the upstream area of the VSG gene ES promoter reproducibly yielded lower CAT gene expression (Fig. 3 A).

Figure 4. Relative activity of VSG, parp and ribosomal promoter in transiently transfected procyclic trypanosomes. CAT activity in relation to the amount of DNA transfected (in pmoles) is depicted for the VSG gene ES promoter (pRK7(+)), the ribosomal RNA genes promoter (pRK7(+)) and the parpA promoter (pF6). Transfections were performed at a constant total DNA concentration of 100 \(\mu g\) per ml (see Materials and Methods). CAT activities for the constructs are in units \(E. coli\) CAT \((\times 100)\) per \(1 \times 10^7\) trypanosomes and are for one representative experiment.

To assess the role of the sequences downstream of the VSG gene ES start sites, restriction fragments around these start sites were subcloned into the parpA splice acceptor CAT gene chimeric constructs. Figure 3 B shows that a 137 bp HaeIII fragment (H2) containing the region −60 to +77 yielded high CAT activities, suggesting that sequences downstream of +77 are not necessary for promoter activity. The significance of the transcription start site at +39 (see Fig. 3 C) was evaluated by deleting an additional 44 bp at the 3' end of this HaeIII fragment with EcoRV. CAT activities significantly above those of promoterless constructs were obtained with this 93 bp H- EV fragment (from −60 to +33). The low activity of this construct was not due to inefficient processing of the primary transcript, because activity was raised 9-fold by adding upstream sequences (fragment EV in Fig. 3 B, from −1137 to +33). The upstream sequences by themselves had no promoter activity (fragments EV-Sc and H1 in Fig. 3 B). Taken together these results show that the −60 to +77 segment is sufficient for high promoter activity. The second start at +39 is not essential for promoter activity, but in the absence of the +33 to +77 segment, sequences upstream of −60 are required for high promoter activity. These upstream sequences have no detectable effect on the −66 to +126 construct.

The promoter region for the ribosomal transcription unit

The ribosomal RNA genes behave as part of a single 10 kb transcription unit in UV inactivation of transcription experiments\(^22\). The putative initiation site for this transcription unit was determined by run-on transcription in isolated nuclei and SI nuclease protection analyses\(^14\) (see Fig. 1 A). We fused a cloned fragment containing the start site to the CAT gene as delineated in Figure 2. The results in Table I (pRK7(+)) show that the region from −258 to +261 bp relative to the 5' end of the putative transcription initiation site (at +1) was sufficient to drive CAT gene expression. The CAT activity obtained was remarkably high, given the inability of pol I in higher eukaryotes to yield functional mRNAs\(^29,30,31\).

Figure 4 shows that the rRNA gene promoter region yielded similar CAT activities as the VSG gene ES and parpA gene promoters in the same splice acceptor CAT construct (Fig. 2). No trypanosome sequences other than the promoter fragments were included in these constructs to avoid the possible

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\(^1\)described in Materials and Methods; region is expressed in bp relative to the transcription start site at +1; correct (+) or reverse (−) orientation relative to the CAT gene; M: transversion-mutant;

\(^2\)[CAT activity in units] \(\times 10^2\) (see Materials and Methods) per \(1 \times 10^7\) trypanosomes. Series A, B and C are CAT activities for one transfection experiment. Each series has been tested at least three times, with variations in absolute CAT activities of about two-fold (compare A and C); −: not determined.
complication of readthrough from trypanosome sequence derived (cryptic) promoters in, for example, 3' untranslated regions necessary for proper polyadenylation (unpublished results).

The rRNA gene promoter was further dissected in experiments summarized in Table I. Sequences downstream of the transcription start site in the rRNA promoter, from +11 to +261 bp, were non-essential. This suggests that the previously identified 5' end of a ribosomal RNA precursor at +115 is most likely an artefact of the nuclease protection analyses, a possibility already raised by White et al. In analogy to other eukaryotic Pol I promoters (reviewed in ref. 32), there seem to be upstream control elements in the T.brucei ribosomal promoter, as deletion of the sequences from −258 to −181 bp resulted in a 7-fold drop in CAT activities. A further deletion of sequences to −75 bp diminished the level of transcription another 7-fold. The −75 to +10 bp region contains all the sequence elements necessary for low-level expression (Table I and Fig. 5). The 5' end of the longest protected fragment (at +1) has limited homology to promoters for RNA polymerase I in other eukaryotes. To test the importance of some of these conserved nucleotides we made single transversion mutants for the G at −16, the A at −7, the T at −1, and the A at +1 in the construct containing the promoter region from −200 to +10. Whereas the −16 and −7 point mutants yielded CAT activities not significantly different from those for the wildtype promoter, the −1 and +1 mutations reduced CAT expression 10-fold (Table I). The dramatic effects on CAT gene expression of specific point mutations at +1 and −1 support the notion of the presence of a specific promoter, with the transcription startpoint at +1.

Comparison of the ribosomal transcription unit promoters of T.brucei and Crithidia fasciculata

Pol I transcription displays a stringent species specificity. The rRNA gene promoters in all but the most closely related species share little DNA sequence similarity. To verify this for the order Kinetoplastida (family Trypanosomatidae), we cloned the transcription initiation region of the rDNA of the kinetoplastid Crithidia fasciculata in the same construct in which the T.brucei rRNA promoter was tested (see Fig. 2). This region extending to −500 bp from the putative transcription start site does not show sequence similarity with the T.brucei rRNA promoter and did not yield CAT activities significantly above background (data not shown).

DISCUSSION

Previous work by us and by Pays and coworkers has shown that the promoter areas from different VSG gene ESs are very similar. Nevertheless, only one promoter out of several is active at a time and this differential promoter control probably occurs at transcription initiation. When the trypanosome enters the tse-tse fly the VSG gene ES promoter remains active, but synthesis of VSG mRNA is prevented by attenuation of transcription. The transected VSG gene ES promoters in procyclic trypanosomes are active as well and can thus be dissected in transient transfections. Earlier transient transfection experiments had already shown that a relatively short segment in front of the transcription initiation site is sufficient for high promoter activity for three different ESs. Hence, we expect that the results of our dissection of the 221 ES promoter will apply to VSG gene ESs in general. The fact that we study the ES promoter in a circular plasmid rather than in its proper location within 100 kb of a chromosome end probably does not grossly affect its behaviour. We have targeted, via homologous recombination, a construct containing the 221 ES promoter fused to a neomycin phosphotransferase (neo') gene into the tubulin gene array (in reverse orientation). This transformant yielded G418-resistant procyclic trypanosomes (unpublished results), showing that the ES promoter can function in a chromosome-internal position.

The mapping of the transcriptional start of VSG gene ESs in vivo has been complicated by the rapid processing of nascent RNA. Using RNase protection we previously mapped a minor start at +1 and a major start at +39 in two ESs. Pays et al. found a single start at +41 (relative to our ES map) in a third ES. After irradiation of trypanosomes with UV light, which inhibits RNA processing, the +39 start became even more prominent, arguing against the idea that this putative start was an early RNA processing site rather than a true start of transcription. Our transfection experiments do not resolve whether there are one or two start sites. Although high promoter activity was obtained with constructs from which the +39 site had been deleted (construct H-EV in Fig. 3 B), these required more upstream sequences for high activity than constructs that contained the +39 site (compare H-EV and H2 in Fig. 3 B). Dissection of the promoter in stable transformants, which allows precise mapping of nascent transcripts, will be required to decide whether sequences at and around +39 contain an efficient start, a pre-mRNA stabilizing motif, an enhancer element, or a combination of these.

Our transfection experiments with wild-type and mutant versions of the transcription initiation region of the rDNA genes in T.brucei show that the ribosomal promoter can drive the synthesis of RNA that is efficiently converted into translatable mRNA. This is remarkable, because analogous experiments in higher eukaryotes have invariably yielded negative results. The mutation experiments presented in Table I suggest that transcription in our transfection constructs starts at the same position as pre-rRNA synthesis in vivo. During transient expression the level of RNA synthesis is too low, however, to reliably analyse RNA synthesis in isolated nuclei for α-amanitin resistance or to study initiation and processing. We have therefore
targeted, via homologous recombination\cite{15}, a single neo\textsuperscript{-}gene driven by the ribosomal promoter into a ribosomal repeat array of \textit{T.brucei}. This stable transformant produces properly transcribed and polyadenylated neo mRNAs\cite{37}. Synthesis of the precursor for this mRNA starts at the ribosomal promoter identified here and by White et al.\cite{14} and is insensitive to 1 mg \textit{\alpha}-amanitin per ml. Hence, our results establish that \textit{T.brucei} has the unusual ability to efficiently synthesize mRNAs derived from transcripts produced by pol I. This removes a major objection against the proposed role of pol I in the \textit{\alpha}-amanitin resistant transcription of the genes for major surface antigens in \textit{T.brucei}.

Whether VSG and procyclin (parp) genes are actually transcribed by pol I rather than by a modified pol II remains to be demonstrated. We have attempted to study this by competition of the different promoters described here, but were unable to saturate the transient expression system with template DNA (see Fig. 4). Sequence gazing did not help either. We detect no homology between the short sequences required for the promoter function of the three known \textit{\alpha}-amanitin-resistant transcription units, VSG (this paper and ref. 1, 24, 26, 27), rRNA (this paper and ref. 14) and procyclin (parp) genes (ref. 8, 20, 24). The several distinct elements usually located upstream of the start of transcription for (class II) protein-coding genes in eukaryotes\cite{38,39}, such as TATA- and CCAAT-boxes, but also those motifs involved in 'TATA-less' transcription initiation\cite{40} appear to be absent in the VSG gene ES promoter.

Whatever the polymerase that transcribes these genes, it is clear that versatile transcription factors have to direct the transcription machinery to diverse start sites. Either separate factors bind to different promoter sequences, but interact with a common RNA polymerase, or a common factor can detect sequence homology that eludes us. Understanding the mechanism by which these transcription factors work together with an RNA polymerase to select sequences as sites of initiation will require the identification of these trans-acting factors and reconstitution of faithful transcription initiation \textit{in vitro}. Thusfar, the trypansom has successfully resisted all attempts to accomplish this.

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