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One- and two-hybrid analysis of the interactions between components of the *Trypanosoma cruzi* spliced leader RNA gene promoter binding complex

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ABSTRACT

The spliced leader (SL) RNA gene promoter is the only RNA polymerase II-dependent promoter characterized to date in trypanosomatids. Transcription of this small nuclear RNA is critical for trypanosomatid cell life because it is needed for polycistronic primary transcripts processing into individual translatable mRNAs. In recent years, a set of divergent fundamental transcription factors required for SL RNA gene transcription have been identified in different trypanosomatids. By means of a yeast two-hybrid system, we analyzed the protein–protein interactions between components of the SL RNA gene promoter binding complex. We also studied the interactions of already described motifs of TATA-binding protein (TBP) and transcription factor II B (TFIIB) orthologs separately. This was followed by investigations of DNA-protein interactions within the SL RNA gene promoter binding complex using one-hybrid analysis. Our results suggest that the complex has two "cores" which contact the promoter DNA, trypanosomal small nuclear RNA activating protein complex (tSNAPc), which has strong interactions between its subunits and a more labile TBP-TFIIA sub-complex.

1. Introduction

Trypanosomatids are a group of early diverged protozoa that cause severe – often lethal – diseases in humans, including leishmaniasis, sleeping sickness and Chagas' disease. They share a particular gene expression mode which differs greatly from the paradigms of eukaryotic gene expression. In these unicellular eukaryotes, protein-coding genes are transcribed polycistronically and then primary transcripts are processed into individual mRNAs by spliced leader (SL) *trans*-splicing and polyadenylation (Perry and Agabian, 1991; Ullu et al., 1993; Campbell et al., 2003). Since unspliced mRNAs are rapidly degraded, trypanosomes need to synthesize large amounts of SL RNA for the production of mature mRNAs, which is achieved by RNA polymerase II (RNAPII)-dependent monocistronic transcription from the multiple SL RNA gene copies which the cell harbors. The SL RNA gene promoters are the only RNAPII-dependent promoters identified to date in trypanosomatids. SL RNA gene promoter sequences differ from one kinetoplastid species to another. In particular, *Trypanosoma cruzi* was described as having only one critical *cis*-element, in contrast to all other studied species which showed at least two *cis*-elements (Campbell et al., 2000a).

Trypanosomatid transcription factors remained uncharacterized until very recently and most of the currently available information concerns SL RNA gene transcription. The first SL RNA gene promoter binding protein was identified in *Leptomonas seymouri* and named PBP-1 for Promoter Binding Protein-1. This protein complex was described as being composed of three polypeptides of 57, 46 and 36 kDa (Luo and Bellofatto, 1997). The 57 kDa protein showed similarity to SNAP50, the 50 kDa subunit of small nuclear RNA (snRNA)-activating protein complex (SNAPc), one of the few mammalian transcription factors found both in RNAP II- and RNAP III-dependent snRNA gene promoters (Das and Bellofatto, 2003). The 46 kDa component seemed to be a protein unique to kinetoplastids, however later identification of the *Trypanosoma brucei* ortholog (TbSNAP42/TbSNAP2) demonstrated the presence of a Myb-like domain, a motif present in the SNAP190 subunit of the human complex, so it is not yet clear whether they represent highly divergent orthologs (Das et al., 2005; Schimanski et al., 2005). In parallel, predicted TbSNAP42/TbSNAP2 folding resembles the bacterial double-stranded DNA-binding protein MutH (Das et al., 2005). Ruan et al. (2004) described a *T. brucei* factor related to the TATA box-binding protein (TbTBP), with about 31% identity to the TBP core domain. This TBP ortholog (also called TBP-related factor 4 (TRF4)) is essential for RNAP I, II and III transcription and is recruited to the SL RNA gene promoter, as well as to RNAP I-transcribed procyclic acidic repetitive genes and RNAP III-transcribed uracil-rich small nuclear RNA and 7SL RNA genes

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(Ruan et al., 2004; Das et al., 2005). Recruitment of trypanosomatid TBP to the SL RNA gene promoter was also demonstrated for *Leishmania tarentolae* and *T. cruzi* (Thomas et al., 2006; Cribb, 2007, PhD Thesis "Characterization of *Trypanosoma cruzi* basal transcription factors", Universidad Nacional de Rosario, Argentina).

Additional components of the SL RNA gene promoter binding complex were isolated from *T. brucei* by Tandem Affinity Purification (TAP) of tagged TbSNAP50 and TbTBP/TbTRF4 (Das et al., 2005; Schimanski et al., 2005). A newly identified 26 kDa SNAP subunit (TbSNAP26/TbSNAP3), although smaller, proved to be the ortholog of the 36 kDa subunit previously identified in *Leptomonas*, and appears to be an internally deleted version of metazoan SNAP43. A *T. brucei* ortholog of TOA2/TFIIA-γ, the smallest subunit of TFIIA, (TbTFIIA2/TbTFIIA-γ) and a 63 kDa protein which probably corresponds to a highly divergent ortholog of the largest TFIIA subunit (TOA1), which is proteolitically cleaved in higher eukaryotes to TFIIAα/β (Tbp63/TbTFIIA1), were also identified as members of the promoter binding complex in *T. brucei*. Additional evidence showed that trypanosomatid TBP/SNAP/TFIIA forms a stable complex essential for SL RNA gene transcription by RNAP II (Das et al., 2005; Schimanski et al., 2005). A highly divergent ortholog to TFIIB also proved to be a component of the SL RNA gene promoter binding complex in *T. brucei* (Schimanski et al., 2006; Palenchar et al., 2006). Trypanosomatid TFIIB showed limited sequence homology to eukaryotic TFIIB and archaeal TFB, but harbors conserved residues within the N-terminal zinc ribbon domain, the B finger and the first cyclin-like repeat. Finally, five genes encoding four putative core TFIIH subunits (TbXPD, TbXPB, Tbp44, Tbp52) and TbXPBz, a novel XPB variant, were described in *T. brucei* (Lee et al., 2007; Lecordier et al., 2007). The TbXPD subunit proved to be essential for SL RNA gene transcription and, as in other eukaryotes, the *T. brucei* TFIIH complex was shown to be involved in DNA repair, cell cycle control and transcription.

As mentioned above, most of the current knowledge about transcriptional control in trypanosomatids comes from studies on SL RNA gene expression in *T. brucei* and *Leptomonas*. At present there is no available data concerning the direct interactions between the components of this complex. Here, we present a detailed analysis of the protein–protein and DNA-protein interactions between the components of the transcription initiation complex formed around the SL RNA gene promoter of *T. cruzi*.

2. Materials and methods

2.1. Yeast strains

Yeast two-hybrid (Y2H) assays were performed using *Saccharomyces cerevisiae* Mav 203 strain (*MAT*α, *leu*2-3,112, *trp*1-901, *his*3-Δ200, *ade*2-101, *gal*4Δ, *gal*80Δ, *SPAL*10::*URA*3, *GAL1::lacZ*, $HIS3_{UAS\,GAL1}$::*HIS3*, *LYS2*, $can1^{R}$ and $cyh2^{R}$) according to the protocol previously described (http://www.invitrogen.com/content/sfs/manuals/10835031.pdf). This yeast strain contains three stably integrated single copy GAL4-inducible reporter genes ($HIS3_{UAS-GAL1}$::*HIS3*, *GAL1*::*lacZ* and *SPAL10*::*URA3*). As protein interaction controls, five derivatives of MaV103 yeast strain (A–E), which have the same genotype as MaV203 except they are *MAT*a, were used. These control strains contain plasmid pairs expressing fusion proteins of increasing interaction strengths: A, no proteins; B, human RB (M28419) amino acids 302–928/human E2F1 (M96577) amino acids 342–437; C, *Drosophila* DP (X79708) amino acids 1–377/*Drosophila* E2F (U10184) amino acids 225–433; D, rat cFos (X06769) amino acids 132–211/mouse cJun (X12761) amino acids 250–325; E, full-length GAL4 (K10486) amino acids 1–881/–.

One-hybrid (Y1H) assays were performed on a reporter strain carrying the −150/+1 region of the *T. cruzi* SL RNA gene promoter integrated upstream of the reporter gene *his*3 into the non-essential locus *pdc6* from the yeast genome. This reporter strain was constructed as described earlier on a *S. cerevisiae* Y187 (*MAT*α *ura3-52his3-Δ200 ade2-101 trp1-901 leu2-3, 112 met⁻ gal4Δ gal80Δ URA3::GAL1*$_{UAS}$-*GAL1*$_{TATA}$-*lacZ MEL1*) background (Ouwerkerk and Meijer, 2001). Briefly, the −150/+1 region of the SL RNA gene promoter was cloned into the pHIS3-NX vector which contains a multiple cloning site upstream of the *his*3 gene. Digestion of this plasmid with restriction enzymes released the (SL(−150/+1)::*his*3) construction, which was subcloned into the pINT1 vector, a second plasmid with *pdc6* flanking sequences, to allow subsequent integration into the yeast genome after transformation with the linearized plasmid. In parallel, a control strain was similarly constructed using an empty pHIS3-NX vector. This control strain (NX::*his*3) bears the same plasmid-DNA sequences as the reporter strain but lacks the SL(−150/+1) sequence upstream of the *his3* reporter gene.

2.2. Cloning of T. cruzi SL RNA gene promoter binding proteins

The *T. cruzi* open reading frames (ORFs) corresponding to the putative subunits of the SL RNA gene promoter binding complex were PCR-amplified with primers designed according to the *T. cruzi* Genome Project sequences (Table 1 and Supplementary Table S1) and directionally cloned into the Gateway entry vector pENTR 3C (Invitrogen). These ORFs were then transferred using LR Clonase reaction to pGBKT7-Gw and pGADT7-Gw Y2H vectors (Clontech), which were previously adapted to Gateway Technology by the introduction of the Gateway cassette to allow cloning by recombination. Once transformed into yeast cells, these plasmids express fusion proteins to the GAL4 DNA-binding (DB) or activation (AD) domain, under control of the constitutive *ADH1* promoter.

2.3. Two-hybrid assays

All X_DB fusion protein-expressing vectors (pGBK-X) were transformed into the MaV203 yeast strain, where "X" represents each one of the SL RNA gene promoter binding complex members or the individually analyzed TcTBP and TcTFIIB domains. Each MaV203/pGBK-X strain was first transformed with the pGADT7 vector and these X_DB/AD containing yeast cells were tested for their ability to activate reporters' expression without a real X-Y protein–protein interaction (self-activation) and to determine the optimal 3-amino-1,2,4-triazole (3AT) concentration required to titrate the basal *his*3 reporter expression to be used later in the interaction assays. MaV203/pGBK-X strains were then transformed with each pGAD-Y construction for Y_AD fusion protein expression (again, Y represents each tested protein). Transformations were performed by electroporation. Briefly, an overnight yeast culture was washed twice with cold sterile water and once with cold 1 M Sorbitol. The cells were resuspended in 1 M Sorbitol, incubated with the appropriate plasmid-DNA and electroporated with a single 2.2 kV pulse. After 1 h incubation in (50:50) 1 M Sorbitol: yeast-peptone-adenine-dextrose medium (YPAD) at 30°, cells were plated in the corresponding minimal selective medium (Synthetic Complete medium (SC) without leucine (-leu) for pGAD-Y transformants, SC without tryptophan (-trp) for pGBK-X transformants and SC-leu-trp for both plasmids transformants).

Once obtained, the Mav203/pGBK-X/pGAD-Y transformants were tested for the three reporter genes' expression phenotypes. Four clones of each X_DB/Y_AD containing yeasts, four DB_X/AD (self-activation control) and two isolated colonies of each of the five yeast control strains were patched in the same SC-leu-trp plate and incubated for 18 h at 30 °C ("Master Plate"). This Master Plate was then replica plated to SC-leu-trp-his+3AT (3AT was added in the optimal concentration previously determined for each X_DB) and to SC-leu-trp-ura to test for growth in absence of histidine