Spliced Leader RNA Gene Transcription in *Trypanosoma brucei* Requires Transcription Factor TFIIH

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Trypanosomatid parasites share a gene expression mode which differs greatly from that of their human and insect hosts. In these unicellular eukaryotes, protein-coding genes are transcribed polycistronically and individual mRNAs are processed from precursors by spliced leader (SL) trans splicing and polyadenylation. In trans splicing, the SL RNA is consumed through a transfer of its 5'-terminal part to the 5' end of mRNAs. Since all mRNAs are trans spliced, the parasites depend on strong and continuous SL RNA synthesis mediated by RNA polymerase II. As essential factors for SL RNA gene transcription in *Trypanosoma brucei*, the general transcription factor (GTF) IIB and a complex, consisting of the TATA-binding protein–related protein 4, the small nuclear RNA-activating protein complex, and TFIIA, were recently identified. Although *T. brucei* TFIIA and TFIIIB are extremely divergent to their counterparts in other eukaryotes, their characteristic suggested that trypanosomatids do form a class II transcription preinitiation complex at the SL RNA gene promoter and harbor orthologues of other known GTFs. TFIIH is a GTF which functions in transcription initiation, DNA repair, and cell cycle control. Here, we investigated whether a *T. brucei* TFIIH is important for SL RNA gene transcription and found that silencing the expression of the highly conserved TFIIH subunit XPD in *T. brucei* affected SL RNA gene synthesis in vivo, and depletion of this protein from extract abolished SL RNA gene transcription in vitro. Since we also identified orthologues of the TFIIH subunits XPB, p52/TFB2, and p44/SSL1 copurifying with TbXPD, we concluded that the parasite harbors a TFIIH which is indispensable for SL RNA gene transcription.

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TFIIIE, TFIIF, and TFIIH form the transcription preinitiation complex (PIC), recruit RNA Pol II to the correct transcription initiation site, separate the DNA strands, and mediate the polymerase’s escape from the promoter (13). Although PIC formation is of fundamental importance to eukaryotes, identification of TFIIA and TFIIB in *T. brucei* was surprising because, except for the multifunctional TRF4 (28) and three putative TFIIH subunits, which in other organisms are known to function beyond PIC formation in DNA repair and cell cycle control, GTFs were not identified by the initial annotation of trypanosomatid genomes (15). The apparent lack of both GTFs and class II promoters for protein-coding gene transcription (22) as well as observations of seemingly nonspecific transcription initiation by RNA Pol II on chromosomal and extrachromosomal DNA (6, 37) fit well with the notion that RNA Pol II-mediated transcription of trypanosomatid protein-coding genes is not promoter driven (22, 34). Nevertheless, the finding that highly divergent TFIIA and TFIIH proteins are involved in SL RNA gene transcription suggested that trypanosomatid genes do form a PIC at the SL RNA gene promoter. If so, SL RNA gene transcription may depend on additional GTFs, such as TFIIH.

TFIIH consists of at least nine subunits and is recruited into the PIC after RNA Pol II. The factor is involved in DNA strand separation at the transcription initiation site and in phosphorylation of the carboxy-terminal domain of the largest RNA Pol II subunit, RPB1 (reviewed in reference 39). Accordingly, eukaryotic TFIIH consists of two subcomplexes: the core complex, which consists of four to five subunits that include the DNA helicase *Xeroderma pigmentosum* B (XPB) and p52, and the cyclin-activating kinase complex, which contains cyclin-dependent kinase 7. Both subcomplexes are linked by the DNA-RNA helicase XPD and its regulator, p44. Trypanosomatid genomes encode clear orthologues of XPB and p52, whereas the claimed identification of cyclin-dependent kinase 7 by sequence homology (15) is ambiguous (12, 23).

Here, we show that silencing of XPD gene expression in *T. brucei* affects SL RNA synthesis in vivo and in vitro, that XPD in extract binds specifically to the SL RNA gene promoter, and that XPD is essential for SL RNA gene transcription in a crude cell extract. Moreover, the finding that the orthologues of the TFIIH subunits XPB, p44, and p52 copurified with XPD and that p52 localized to the nucleus confirmed that *T. brucei* harbors a TFIIH which is required for SL RNA gene transcription.
to the culture medium at a final concentration of 10000 cells/ml. The \( H_{2} O_{2} \) were diluted to 30% phleomycin. Silencing of TbXPD of the SacII-linearized construct pTbXPD-stl and cloned by limited dilution in \( H_{2} O_{2} \) with StuI and transfected into TbX1 cells. The selection conditions were 200 ng/ml of hygromycin, 40 \( H_{2} O_{2} \), and 3 \( H_{2} O_{2} \)-tubulin, respectively. Template DNAs SLIns19 and GPEET-trm have been described previously (8, 19), but since both plasmids contained T7 promoters which were incompatible with extracts of T7 RNA Pol-expressing 29-13 cells, the template DNAs were recloned into vector pUC19 using restriction sites EcoRI and HindIII for SLins19-\( H_{2} O_{2} \) and KpnI and EcoRI for GPEET-trm-\( H_{2} O_{2} \).

Cells. Prokaryotic cell culture, targeted integration of linear DNAs into cells by electroporation, and the generation of stable cell lines by selection and limiting dilution were done as previously described (9, 10). The cell line TbX1 was generated by replacing one \( H_{2} O_{2} \) allele in the clonal cell line TbX1. One allele was replaced by the hygromycin phosphotransferase gene (HYG-R), while the vector pXPD-PPT-NEO was inserted into the second allele. The \( H_{2} O_{2} \) coding region is represented by open boxes, the \( H_{2} O_{2} \) tag by a black box, resistance marker coding regions by striped boxes, and introduced gene flanks for RNA processing signals by smaller gray boxes. (B) Immunoblot monitoring of the XPD-PPT purification. Aliquots of input material, the flowthrough of the IgG columns (FT-IgG), the TEV protease eluate (Elu TEV), the flowthrough of the anti-ProtC column (FT-ProtC), and final EGTA (Etu EGTA) and peptide (Etu Pep) elutions were separated on a 10% SDS-PAGE and detected with a monoclonal antibody directed against the ProtC epitope. Values followed by \( H_{2} O_{2} \) indicate relative amounts of each fraction analyzed. Marker sizes are depicted on the left, and the size difference between the full-length XPD-PPT and the TEV protease-cleaved XPD-P is indicated on the right. (C) Total eluate of the TbXPD-PPT tandem affinity purification was separated on a 10 to 20% SDS-polyacrylamide gradient gel and stained with Coomassie blue. For comparison, 0.002% of the input material and 5% of the TEV protease eluate (Etu TEV) were loaded. The four unambiguously identified proteins are specified on the right, whereas protein marker sizes are listed on the left.

MATERIALS AND METHODS

Plasmids. pTbXPD-stl is a derivative of the \( T. \) brucei stem-loop RNAi vector (1, 33) containing the TbXPD region from position \( H_{2} O_{2} \) to \( H_{2} O_{2} \) relative to the translation initiation codon as inverted repeats around a stuffer sequence. pXPD-PPT-NEO was derived from pC-PTP-NEO (32) by fusing the C-terminal 234 bp of the TbXPD-PTP tandem affinity purification was separated on a 10 to 20% SDS-polyacrylamide gradient gel and stained with Coomassie blue. For comparison, 0.002% of the input material and 5% of the TEV protease eluate (Etu TEV) were loaded. The four unambiguously identified proteins are specified on the right, whereas protein marker sizes are listed on the left.

FIG. 2. PTP purification of TbXPD. (A) Schematic depiction of the two TbXPD alleles in the clonal cell line TbX1. One allele was replaced by the hygromycin phosphotransferase gene (HYG-R), while the vector pXPD-PPT-NEO was inserted into the second allele. The TbXPD coding region is represented by open boxes, the \( H_{2} O_{2} \) tag by a black box, resistance marker coding regions by striped boxes, and introduced gene flanks for RNA processing signals by smaller gray boxes. (B) Immunoblot monitoring of the XPD-PPT purification. Aliquots of input material, the flowthrough of the IgG columns (FT-IgG), the TEV protease eluate (Elu TEV), the flowthrough of the anti-ProtC column (FT-ProtC), and final EGTA (Etu EGTA) and peptide (Etu Pep) elutions were separated on a 10% SDS-PAGE and detected with a monoclonal antibody directed against the ProtC epitope. Values followed by \( H_{2} O_{2} \) indicate relative amounts of each fraction analyzed. Marker sizes are depicted on the left, and the size difference between the full-length XPD-PPT and the TEV protease-cleaved XPD-P is indicated on the right. (C) Total eluate of the TbXPD-PPT tandem affinity purification was separated on a 10 to 20% SDS-polyacrylamide gradient gel and stained with Coomassie blue. For comparison, 0.002% of the input material and 5% of the TEV protease eluate (Etu TEV) were loaded. The four unambiguously identified proteins are specified on the right, whereas protein marker sizes are listed on the left.

![Image](https://example.com/image.png)
FIG. 3. Sequence alignment of TFIIH p44 and p52 orthologues. (A) ClustalW alignment of p44/SSL1p sequences from Homo sapiens (Hs; accession number NP_001506), Drosophila melanogaster (Dm; NP_649427), Caenorhabditis elegans (Ce; NP_499239), Arabidopsis thaliana (At; NP_683275), and Saccharomyces cerevisiae (Sc; YLR005W), as well as from the trypanosomatids Trypanosoma brucei (Tb; GeneDB accession number Tb927.8.6540), Trypanosoma cruzi (Tc; Tc00.1047053511907.300), Leishmania major (Lm; LmjF24.1680), and Leishmania infantum (Li; LinJ10.0190). Identities and similarities are shaded in black and gray, respectively. Only positions with a minimum of five identical or conserved
with the C-terminal C4C4 RING domain. aa, amino acids. Identity/similarity values specified at the end of each sequence were determined by pair-wise alignment of the C-terminal region of p52/TFB2 sequences. Identity and similarity values were calculated from the complete sequences. Sequence accession numbers are NP.001508 (Hs), NP.648780 (Dm), NP.502859 (Ce), NP.974564 (At), YPL122C (Sc), Tb10.70.1900 (Tb), Tc00.1047053510297.80 (Tc), LmjF36.0800 (Lm), and LinJ36.1830 (Li).

RESULTS AND DISCUSSION

Silencing of TbXPD gene expression affects SL RNA synthesis in vivo. The aim of this study was to assess whether T. brucei harbors TFIIH and whether this factor is involved in SL RNA gene transcription. Of the 10 known TFIIH subunits, homologous sequences of only two subunits, the helicases XPD and XPB, have been unambiguously identified (2). XPD is encoded by a single gene (GeneDB accession number Tb927.8.5980), whereas there are two genes which encode putative homologues of XPB: the predicted proteins of genes Tb927.3.5100 and Tb11.01.7950 have calculated masses of 105 kDa and 89 kDa, respectively, and are highly divergent to each other and to XPBs of other organisms (data not shown), the significance of which is not understood. We therefore chose to begin our analysis with TbXPD. At first, we employed the conditional RNAi system to silence the expression of TbXPD in procyclic trypanosomes. Expression, in double-stranded TbXPD RNA was induced by adding doxycycline to the medium. While induced cells grew nearly normal up to 72 h, they subsequently ceased growth and died rapidly (Fig. 1A). Since we observed this effect with three independently generated cell lines and since the induction of double-stranded RNA (dsRNA) synthesis clearly affected the abundance of XPD RNA (Fig. 1B), we concluded that TbXPD is encoded by an essential gene.

Next, we used a primer extension assay to compare the amounts of SL RNA and U2 snRNA in total RNA of cells in which XPD expression was silenced. In T. brucei, U2 snRNA synthesis is mediated by RNA Pol III and, therefore, should not depend on XPD/TFIIH. Accordingly, XPD silencing for 48 and 72 hours decreased the abundance of SL RNA to 17% and 13%, respectively, whereas U2 snRNA amounts were not significantly affected (Fig. 1C). Primer extension of SL RNA typically results in two extension products because, in contrast to newly synthesized SL RNA, mature SL RNA has a unique cap4 structure with unusual methylation of the first four nucleotides which cause premature termination of reverse transcription. Interestingly, the larger extension product detecting unmethylated SL RNA was more strongly reduced than the product of the mature form, suggesting that XPD is involved in SL RNA synthesis rather than in maturation. To more directly analyze the effect of XPD silencing on SL RNA gene transcription, we employed a permeabilized cell system to radio-label nascent RNA (35). Since the SL RNA is synthesized at a very high rate, it can be directly visualized by autoradiography, between high-molecular-mass pre-rRNA/pre-mRNA and tRNA, when total RNA is separated by denaturing PAGE (Fig. 1D) (8, 35). Labeling of the SL RNA was reproducibly decreased by approximately 60% in cells in which XPD RNAi was induced for 48 or 72 h. While this effect seems moderate, it was comparable to the reduction caused by RNAi of the essential factors TRF4 (28) and TFIIIB (29), raising the possibility that reducing SL RNA synthesis to this extent is sufficient to inhibit in vitro growth of procyclic trypanosomes. Our finding that steady-state levels of SL RNA were more strongly affected (~87%) than the synthesis rate of nascent SL RNA (~60%) is in accordance with this hypothesis and suggests that SL RNA turnover by trans splicing is greater than the rate of SL RNA synthesis in cells in which XPD expression was silenced for a minimum of 48 hours. Together, these experiments indicated that XPD plays a role in SL RNA gene transcription.

TbXPD is associated with TFIIH subunit orthologues. As a tool for TFIIH characterization and functional analysis of XPD in vitro, we generated the clonal cell line TbX1, which exclusively expressed XPD C-terminally fused to the PTP tag (Fig. 2A). The latter is a combination of protein A and protein C (ProtC) epitopes separated by a tobacco etch virus (TEV) protease cleavage site and designed for tandem affinity purification of a tagged protein (32). Since we had shown that TbXPD is essential for trypanosome growth and since TbX1 cell growth was not significantly altered when compared to wild-type cells (data not shown), we inferred that the PTP tag did not critically interfere with the function of TbXPD. We therefore prepared a crude cell extract from TbX1 cells which consisted of a mix of cytoplasmic and extracted nuclear components and PTP-purified XPD sequentially by IgG affinity chromatography, TEV protease elution, and anti-ProtC immunofinity chromatography. The protein was finally eluted with either EGTA or the ProtC peptide. Monitoring of the purification by immunoblotting showed that while IgG bound nearly all of the XPD-PTP in the extract, binding of the tagged protein to the anti-ProtC matrix was less efficient (Fig. 2B, compare lanes 1 and 3 with lanes 2 and 4). Due to the latter step, the overall purification efficiency of XPD-PTP was lower than

residues are shaded. Parasite-specific identities are shaded in red. The asterisks mark the conserved C in the central domain and the C-terminal C4C4 RING domain. aa, amino acids. Identity/similarity values specified at the end of each sequence were determined by pair-wise comparison with the T. brucei sequence using the EMBOSS program (http://www.ebi.ac.uk/emboss/align/) at default settings. (B) Analogous alignment of the C-terminal region of p52/TFB2 sequences. Identity and similarity values were calculated from the complete sequences. Sequence accession numbers are NP.001508 (Hs), NP.648780 (Dm), NP.502859 (Ce), NP.974564 (At), YPL122C (Sc), Tb10.70.1900 (Tb), Tc00.1047053510297.80 (Tc), LmjF36.0800 (Lm), and LinJ36.1830 (Li).
that of previously reported PTP-tagged proteins (24, 26, 31, 32). Nevertheless, SDS-PAGE and Coomassie staining of the final eluate resulted in the detection of 10 distinct protein bands (Fig. 2C). Liquid chromatography-tandem mass spectrometry analysis revealed four clear orthologues of TFIIH subunits in the final eluate thus far. The top band was identified as the large XPB protein encoded by gene Tb927.3.5100 and the strong band below as XPD-P.

In addition, analysis of the band with an apparent size of 36 kDa identified a protein which is encoded by gene Tb927.8.6540. The predicted size of this protein is 38 kDa, and it has orthologues in the genomes of *Trypanosoma cruzi* and *Leishmania major* (Fig. 3A). A sequence alignment unambiguously identified this protein as the orthologue of the human TFIIH subunit p44 and the yeast counterpart SSL1p (Fig. 3A). The C-terminal domain of this subunit is important for transcription initiation and, as a key feature, harbors a zinc-binding C4C4 RING structure (17). Accordingly, all eight C residues of this structure are conserved in trypanosomatids. The central domain of p44 is involved in the formation of the TFIIH core complex harboring an additional zinc-binding motif which is also perfectly conserved in the trypanosomatid sequences (Fig. 3A).

Furthermore, we identified a protein in the doublet band of 53/55 kDa which is encoded by gene Tb10.70.1900. While this protein was annotated as conserved hypothetical, it exhibited homology to the TFIIH subunit p52, which is termed TFB2 in yeast. p52 binds directly to the helicase XPB, and a mutational analysis showed that its C-terminal domain is involved in both transcription and DNA repair anchoring XPB within the TFIIH core complex (16). Accordingly, a sequence comparison between p52/TFB2 of model organisms and its putative trypanosomatid orthologues revealed the highest degree of conservation in the C terminus (Fig. 3B and data not shown).

Overall, the putative p52 orthologue was less conserved than p44, and it did not contain a clear sequence motif. To unequivocally show that the correct protein was identified, we C-terminally HA-tagged *T. brucei* p52 in TbX1 cells, prepared extract from the new cell line, and conducted reciprocal coimmunoprecipitation assays. Precipitation of XPD-PTP by IgG-coated beads specifically coprecipitated p52-HA and, vice versa, the use of an anti-HA antibody precipitated both p52-HA and XPD-PTP (Fig. 4). This interaction was specific, because the U2 snRNP protein U2-40K, which is predominantly localized in the nucleus (27), did not coprecipitate with either protein. Since we can also exclude a nonspecific interaction between HA and the PTP tag (24), these results clearly confirmed the interaction of XPD and p52.

Finally, we have achieved preliminary identification of five additional XPD copurified proteins (data not shown). All of them have been annotated as conserved hypothetical proteins and, by primary structure, appear to be parasite-specific proteins. We are in the process of confirming these identifications and evaluating the functional significance of each of them.

In summary, PTP purification of TbXPD revealed a minimum of nine copurified proteins, three of which were unambiguously identified as orthologues of TFIIH subunits. This strongly indicated that trypanosomes possess a transcriptionally relevant TFIIH.

The TFIIH subunit p52 localizes to the nucleus. The HA fusion to p52 enabled us to localize this protein in the cell.
Thus far, we have been unable to utilize the protein A and C epitopes of the PTP tag to localize tagged proteins by indirect immunofluorescence. In contrast, the HA epitope has been successfully used for localization studies in trypanosomes. Accordingly and as expected, the use of the monoclonal rat anti-HA antibody specifically stained the nucleus of TbX1 cells which expressed p52-HA (Fig. 5), whereas there was no detectable signal with TbX1 cells not expressing an HA tag (data not shown). This control also demonstrated that the protein A domains of XPD-PTP, expressed in TbX1 cells, did not interact with the antibody at a detectable level. Hence, the nuclear localization of p52 further supported the correct identification of this protein as a subunit of T. brucei TFIIH.

**TbXPD specifically binds to the SL RNA gene promoter.**

Since silencing of TbXPD expression affected SL RNA synthesis in vivo, we wanted to confirm a function of TbXPD in SL RNA gene transcription first by analyzing its interaction with the SL RNA gene promoter. We have previously employed a promoter pull-down assay to demonstrate the specific interaction of all subunits of TRF4/SNAPc/TFIIA and of TFIIH with the SL RNA gene promoter (29, 31). In the same assay, XPD-PTP clearly bound to the SL RNA gene promoter but not to nonspecific DNA or the class I GPEET procyclin gene promoter (Fig. 6, compare lanes 3, 4, and 6). We also included in our analysis an SL RNA gene promoter DNA in which the first motif of the USE was mutated. This mutation abolished binding of the TRF4/SNAPc/TFIIA complex to the SL RNA gene promoter (Fig. 6) (31). In contrast, XPD-PTP bound more efficiently to the mutant than to the wild-type promoter, indicating that TFIIH binding is independent of TRF4/SNAPc/TFIIA (Fig. 6, compare lanes 4 and 5). Furthermore, since the PIC dissociates after transcription initiation (13) and our binding reactions may support transcription initiation, the inability of the mutant SL RNA gene promoter to direct transcription initiation (8) may have stabilized the interaction with XPD-PTP. Together, these results established that, in extract, XPD-PTP is specifically recruited to the SL RNA gene promoter.

**TbXPD is essential for SL RNA gene transcription in vitro.**

In a final step, we analyzed the function of TbXPD in an in vitro transcription system. This system is based on a crude transcription extract which consists of cytoplasmic and extracted nuclear components and which is active for both RNA Pol I-mediated and class II SL RNA gene transcription (19). T. brucei utilizes RNA Pol I not only for transcription of the large rRNA genes as other eukaryotes, but also for those gene units which encode the major cell surface antigens variant surface...
glycoprotein and procyclin (10). Although all class I promoters function in the in vitro system, the GPEET procyclin gene promoter is most effective (19). Hence, we cotranscribed the templates GPEET-trm and SLins19, which harbor the GPEET gene/promoter and SL RNA gene promoter, respectively. Both templates carry unrelated tag sequences downstream of the transcription initiation site, enabling the specific detection of the GPEET-trm and SLins19 RNAs by primer extension assays. To assess the function of XPD for SL RNA gene transcription, we first prepared transcription extract from TbX1 cells which exclusively express XPD-PTP. Subsequently, we depleted XPD from this extract by IgG chromatography (Fig. 7A). In comparison to mock-treated extract, XPD depletion virtually abolished transcription of template SLins19, whereas it did not affect GPEET-trm transcription (Fig. 7B). The transcriptional activity of SLins19 could be partially restored in a dose-dependent manner when the final peptide eluate of the XPD-PTP tandem affinity purification was added back (Fig. 7B, + Elu). This was not a nonspecific effect of the tag, because we have previously shown that PTP-tagged TbSNAP2 and TbRPA1 proteins are unable to stimulate SL RNA gene transcription in TFIIH-depleted extract (29). Hence, these results demonstrated that the observed effects on SL RNA gene transcription were caused by the XPD-PTP purified proteins. They did not, however, unambiguously identify XPD as an essential protein for this activity. To show this, we prepared transcription extract from an XPD-RNAi competent cell line before and 72 h after inducing synthesis of XPD dsRNA. In comparison to the previous experiments, the relative activities of GPEET-trm and SLins19 transcription were different (Fig. 7B and C). This variability was most likely a result of large- and small-scale extract preparations for the depletion and RNAi experiments, respectively. Nevertheless, the signal strengths within each experimental set were consistent. Hence, the results clearly showed that SLins19 transcription was strongly affected by XPD silencing, whereas GPEET-trm transcription was not (Fig. 7C). Since the final peptide XPD-PTP elute fully reconstituted the transcriptional activity, again in a dose-dependent manner, a nonspecific RNAi effect could be excluded. Thus, we concluded that XPD is essential for SL RNA gene transcription in T. brucei.

Conclusion. In this study, we have partially characterized TFIIH of T. brucei and shown that the TFIIH subunit XPD is essential for transcription of the SL RNA gene. After the identification of TFIIA and TFIIIB, this is the third trypanosome GTF playing a crucial role in SL RNA gene transcription. Together, these findings strongly argue that trypanosomes form a conventional class II PIC at the SL RNA gene promoter. If so, it can be expected that the parasites also harbor orthologues of the missing GTFs, TFIIIE and TFIIIF. The surprising aspect of these findings is the extreme divergence in primary structure between mammalian and trypanosomatid GTFs which, in the case of TFIIH, exceeds the divergence between the mammalian and archael orthologues (29). The observed divergence of trypanosomatid sequences may be a result of relaxed evolutionary constraints due to relatively few PIC formation sites in the genome. The extraordinary divergence level offers the promising perspective that trypanosomatids have evolved unique and essential structural and/or biochemical features in a fundamentally important process, namely, the recruitment of RNA Pol II to DNA.

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