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JBP2, a SWI2/SNF2-like Protein, Regulates De-novo Telomeric DNA Glycosylation in Bloodstream Form *Trypanosoma brucei*

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Abstract

Synthesis of the modified thymine base, β -D-glucosyl-hydroxymethyluracil or J, within telomeric DNA of *Trypanosoma brucei* correlates with the bloodstream-form specific epigenetic silencing of telomeric variant surface glycoprotein genes involved in antigenic variation. In order to analyze the function of base J in the regulation of antigenic variation, we are characterizing the regulatory mechanism of J biosynthesis. We have recently proposed a model in which chromatin remodeling by a SWI2/SNF2-like protein (JBP2) regulates the developmental and de-novo site-specific localization of J synthesis within bloodstream-form trypanosome DNA. Consistent with this model, we now show that JBP2 (–/–) bloodstream-form trypanosomes contain 5-fold less base J and are unable to stimulate de-novo J synthesis in newly generated telomeric arrays.

Introduction

In the DNA of kinetoplastid flagellates, a fraction of thymine is replaced by the modified base β -D-glucosyl-hydroxymethyluracil, called J [1–3]. This hyper-modified base consists of the attachment of a bulky glucose moiety to the thymine base such that it extends into the major groove of the DNA helix. While J is abundantly present in telomeric repeats of all kinetoplastids, in the parasite *Trypanosoma brucei* it is also found in the sub-telomeric variant surface glycoprotein (VSG) gene expression sites involved in antigenic variation [3–5]. By periodically switching its VSG coat the trypanosome is able to evade the host immune system in chronic infections. The expressed VSG, which is localized on the surface of the cell, is encoded by a gene transcribed from 1 of about 20 telomeric VSG expression sites. Only one of the 20 expression sites is active at any given time. The presence of J within the ~19 inactive telomeric VSG gene expression sites but not in the active site suggests that J may be involved in the transcriptional repression of VSG gene expression sites and thus, antigenic variation [3, 5, 6].

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Along with the process of antigenic variation, the synthesis of base J is developmentally regulated during the parasite life-cycle. Trypanosome DNA is modified only during infection of the mammalian host (bloodstream lifestage) and not during infection of the midgut of the vector, tsetse fly (procyclic lifestage). In order to analyze the biological function of base J in *T. brucei*, we need to first characterize the factor(s) regulating J-biosynthesis. Substantial indirect evidence [6–8] indicates that the modification of thymine in trypanosome DNA occurs in two steps: a thymine is oxidized to hydroxymethyluracil (HOMeUra) by a putative thymine-7-hydroxylase (TH), followed by glucosylation of the new base by a putative glucosyltransferase (GT). All current data suggests that the stage specific regulation occurs at the first step, since procyclic-form trypanosomes are able to synthesize J when grown in the presence of hydroxymethyldeoxyuridine (HOMeDU), the intermediate in the J-biosynthesis pathway. Therefore procyclic-form trypanosomes do contain an active GT component. While all attempts to isolate TH and GT activities have failed thus far, the identification of a base J binding protein (JBP1) in bloodstream-form *T. brucei* nuclear extracts led to the characterization of the first co-factor involved in J biosynthesis [9]. Analysis of the bloodstream form JBP1 (–/–) trypanosome, and ectopic expression in procyclic-form trypanosomes, has indicated that JBP1 represents a propagation and inheritance factor for J-biosynthesis [10, 11]. JBP1 binds J-DNA and presumably activates (i.e. recruits) the synthesizing machinery to introduce additional J in regions already containing a basal level of J; therefore, spreading J along the chromosome (propagation) or on the opposing DNA strand following replication (inheritance)[11].

We have recently demonstrated that a homologue of JBP1, JBP2, which contains an additional region homologous to the family of SWI2/SNF2-like chromatin remodeling proteins, stimulates de novo site-specific synthesis of a basal level of J by ectopic expression of JBP2 in procyclic form trypanosomes. This can now be further propagated and maintained by concomitant expression of JBP1 [11]. JBP2 (as well as JBP1) is developmentally regulated and interacts with nuclear chromatin. Mutations within the SWI2/SNF2 domain of JBP2 inhibit the stimulation of J-biosynthesis when expressed in procyclic cells [11]. While these previous studies allowed the direct analysis of JBP2 function in a J-minus background, it relied on “over-expression” of JBP2 in a developmental life-stage (procyclic-form trypanosomes) that normally lacks the modified base. Based on these studies we proposed a model where chromatin remodeling by JBP2 represents the critical process for regulating J-biosynthesis in bloodstream form trypanosome DNA. We now directly examine JBP2 function in the bloodstream-form of *T. brucei* by analyzing the JBP2 double KO trypanosome. We find that the JBP2 (–/–) trypanosomes contain 5-fold less J and are unable to stimulate de-novo J-biosynthesis in newly generated telomeric DNA. These results confirm our characterization of JBP2 as the key regulator of J-biosynthesis in bloodstream form trypanosomes: regulating the developmental and site-specific de-novo synthesis of J in the genome.

Results

JBP2 is involved in J-biosynthesis in bloodstream form trypanosomes

To investigate the biological function of JBP2 in bloodstream form trypanosomes, we generated cell lines lacking both JBP2 alleles. This was done using constructs that delete the entire JBP2 ORF, replacing it with a cassette for blasticidin S deaminase (blasticidin resistance) in one allele and with the hygromycin phosphotransferase (hygromycin resistance) gene in the other allele, as shown in Figure 1A and detailed in Experimental Procedures. These two drug resistance cassettes, cloned between JBP2 flanking sequences, enabled replacement of both alleles of the JBP2 gene via two independent rounds of transfection. Southern blot analysis confirmed gene replacement (Figure 1B). To directly verify the absence of JBP2 in the JBP2 (–/–), we raised polyclonal peptide antibodies against JBP2 and tested wild-type and JBP2 (–/–) extracts. As shown in Figure 1C, we see a reactive bloodstream form specific polypeptide of the appropriate molecular weight in wild-type cells which is absent in the JBP2 (–/–). The specificity of the antisera is indicated by the lack of reactive polypeptide using pre-immune serum (Figure 1D) and undetectable levels of the JBP2 specific polypeptide in procyclic cells versus the ability to detect the ectopically expressed JBP2-GFP fusion in procyclic cells.

JBP2 (–/–) trypanosomes were able to grow normally *in vitro* (data not shown). Analysis of the JBP2 (–/–) by IFA and RT-PCR indicate they continue to exclusively express the 221 VSG expression site with no de-repression of the ~19 silent expression sites (data not shown). To look for a phenotype, we examined the levels of base J in genomic DNA of the JBP2 (–/–). We have previously proposed that the basal level of J remaining in the JBP1(–/–) (~5%) is due to JBP2 function in stimulating de-novo J-biosynthesis [11]. Consistent with this idea, the JBP2 (–/–) was found to contain less J than wild-type trypanosomes. This is illustrated by the anti-J DNA dot blot assay, shown in Figure 1E. Genomic DNA was serially diluted and blotted onto nitrocellulose. The amount of J is determined by immunodetection using anti-J antiserum (Figure 1E, left panel). To quantify the amount of DNA loaded on the blot, the filter was stripped, hybridized with a β -Tubulin gene probe and phosphorimaged (Figure 1E, right panel). The DNA of procyclic *T. brucei*, the insect life-cycle stage which completely lacks base J, is included as a negative control. Whereas heterozygous JBP2 (+/–) trypanosomes have about 2-fold less genomic J than wild-type, the homozygous JBP2 (–/–) trypanosomes show a 5-fold reduction in J. Anti-J immunoprecipitation analysis indicates that the reduction in J is not uniform within all J-specific regions of the genome (Figure 1F). Quantitation of the data indicates an average 2–3 fold reduction, compared to WT trypanosomes, in %IP of J-DNA corresponding to these regions with an increase in J loss from telomeres and little to no loss of J within the 177bp repeats. However, this apparent unequal decrease in J throughout the J-specific regions of the genome may be a consequence of the remaining propagation/maintenance activity of JBP1 in the JBP2 (–/–) and not representative of any specificity of JBP2 function. The analysis of JBP2 function in a bloodstream form cell line that lacks both JBP2 and JBP1 (see Discussion) will clarify this situation. To confirm that the reduction in J modification was caused by the loss of JBP2 protein, we examined the effect of re-expressing JBP2 in the JBP2 (–/–) trypanosomes from the tubulin locus. JBP2 (–/–) clones, ectopically expressing

the JBP2-GFP fusion protein were found to restore the level of J to 70–90% of WT levels (Figure 1E and data not shown). The variation in level of base J in the JBP2 rescue cells may be due to differential levels of JBP2-GFP expression from the tubulin array not detected by western blot analysis. It may also reflect limitations of JBP2 function imposed by the remaining high levels of J maintained in the genome by JBP1. This high level of J may interfere with the ability of JBP2-GFP to stimulate the conversion of adjacent thymine residues to J. This would also explain the inability of JBP2-GFP to fully restore the levels of J in the JBP2 (–/–). We conclude from these results that JBP2 is involved in J-biosynthesis in bloodstream-form trypanosomes.

JBP2 stimulates de novo J-synthesis

In order to determine if the reduction in J levels in the JBP2 (–/–) trypanosomes is due to the failure to re-initiate de novo J biosynthesis, we examined if these cells are able to stimulate J synthesis in a newly generated telomere. To do this we took advantage of the telomere-mediated fragmentation construct (pTMF) [12] which allows the generation of a de novo telomere at the active 221 VSG locus (Figure 2A). Southern analysis indicates that the NEO gene is linked to the VSG gene and that adjacent de novo telomeric repeats are rapidly extended in both wild-type and JBP2 (–/–) trypanosomes (Figure 2B). To analyze the new telomeres for the presence of base J, DNA was digested with Xho I and immunoprecipitated with anti-J antisera. The inability to immunoprecipitate the Xho I telomeric fragment would indicate a lack of base J in this specific region of the chromosome [5]. Southern blotting indicates significant precipitation of the Xho I telomeric fragment from wild-type cells and no precipitation from the JBP2 (–/–). Therefore, the new telomeres in wild-type cells contain base J while the telomeric repeats in the JBP2 (–/–) cells lack J (Figure 2C). Hybridization of the blot with a 177bp telomere probe or anti-J antisera indicates the IP reactions precipitated various J-containing DNA fragments in all reactions analyzed (data not shown and Figure 2D). Taking into consideration the 5-fold reduction of J, this indicates that the failure to precipitate the newly generated 221 ES telomere fragment in the JBP2 (–/–) is due to a lack of base J and not failure of the IP reaction itself. This is supported by the finding that the lack of J in newly synthesized telomeres in the JBP2 (–/–) can be rescued by either growing the cells in the presence of HOMedU, or by ectopic expression of the JBP2-GFP fusion protein (Figure 2C). Both HOMedU feeding and JBP2-GFP expression lead to an increase in J levels (Figure 2E). However, HOMedU is randomly incorporated, and converted to J, throughout the genome [6] while JBP2 is expected to stimulate telomeric localized J-biosynthesis [11]. The non-specific increase in J by HOMedU explains the large increase in total levels of J present in the genome relative to that stimulated by JBP2 function. Therefore, while there is significant levels of J in cells fed HOMedU, versus JBP2 expression, there may be differential levels of J synthesized in the specific telomeric fragment analyzed here. From these results, we conclude that JBP2 stimulates de novo J-biosynthesis in bloodstream form trypanosomes.

Discussion

The purpose of this study is to determine the biological function of JBP2 in bloodstream form trypanosomes. Previous studies of JBP2 function using procyclic trypanosomes, the

life-stage which normally lacks J, have implicated it as the key regulator in J-biosynthesis: regulating the developmental life-stage specific and site-specific de novo synthesis of J in the bloodstream form trypanosome genome [11]. Here, we demonstrate that JBP2 is involved in the J-biosynthesis pathway of bloodstream form trypanosomes, regulating de novo J-biosynthesis in genomic DNA.

Trypanosoma brucei and related kinetoplastid flagellates contain the modified base J in their telomeres. In *T. brucei*, the presence of J is developmentally regulated and abundant in repetitive sequences present at sub-telomeric expression sites involved in antigenic variation. In order to elucidate the biological function of base J, we have tried to isolate and characterize proteins involved in regulating J-biosynthesis in the bloodstream form trypanosome. The search for proteins in bloodstream form nuclear extracts, able to bind J containing DNA, led to the identification of the first co-factor involved in J-biosynthesis: JBP1. JBP1 is a J-DNA specific binding protein involved in the maintenance and propagation of base J in the trypanosome genome [9–11, 13]. JBP1 requires residual levels of J in the genome to bind and directly stimulate further synthesis along the DNA (propagation) as well as, presumably, on the opposing strand after replication (inheritance). Thus, ectopic expression of JBP1 in procyclic cells does not lead to the stimulation/propagation of J-biosynthesis unless a basal level of J is provided in the genome [11].

The second identified co-factor involved in J-biosynthesis, JBP2, contains an N-terminus with homology to JBP1 [11]. Although JBP2 was initially identified based on the homology with JBP1, it is also a member of the SWI2/SNF2-like family of chromatin remodeling proteins. The SWI2/SNF2 domain of JBP2 includes the seven sequence motifs that are characteristic of members of the DEAD/H superfamily of nucleic-acid-stimulated ATPases and DNA helicases as well as additional sequence elements that are highly conserved only in members of the SWI2/SNF2 family [11]. JBP2 is developmentally regulated, it associates with chromatin *in vivo*, and when ectopically expressed in procyclic cells, leads to the site-specific synthesis of base J. Concomitant expression of JBP1 then allows further propagation of J within the localized chromatin region containing the basal level of J due to JBP2 function [11]. While the lack of recombinant protein has precluded the *in vitro* analysis of the SWI/SNF motif, the ability of JBP2 to stimulate de novo J synthesis in procyclic cells is inhibited after mutation of residues implicated in ATPase/chromatin remodeling function of SWI2/SNF2 like proteins. Therefore, we have proposed that JBP2 recognizes and binds certain regions of the chromosome, hydrolyses ATP and allows the J-synthesis machinery to gain access to condensed chromatin by altering chromatin architecture [11]. JBP1 then binds to this basal level of localized J and stimulates additional synthesis by recruitment of the machinery to DNA [10, 11].

The analysis of JBP2 function thus far, has been based on over-expression studies in the trypanosome life-stage which completely lack base J. While this provided a clear negative background for J-biosynthesis studies, it did not allow the analysis of JBP2 function in the relevant life-stage. Here we directly examine the biological role of JBP2 in bloodstream form trypanosome by the analysis of the JBP2 (–/–) trypanosome. We show that deletion of both JBP2 alleles leads to a 5-fold reduction of J levels, supporting its proposed role in the J-biosynthesis pathway. To directly examine the ability of JBP2 to stimulate de novo J-

synthesis, we utilized the telomere fragmentation (TMF) assay allowing the generation of new telomeric repeat DNA substrate for J-modification. Using this assay we show that wild-type cells are able to re-initiate J-synthesis in these newly generated telomeres whereas in the JBP2 (–/–) trypanosomes these telomeric regions lack base J. Re-introduction of JBP2 rescues this absence of J. These results support the conclusion that the function of JBP2 in J-biosynthesis is to stimulate a basal level of de novo J-modification within specific regions (primarily telomeric) of bloodstream form *T. brucei* chromatin. JBP1 would then bind and propagate further J-synthesis. The JBP1 (–/–) trypanosome contains 20-fold less J than wild-type cells [10]. Our model of JBP2 function would predict that the basal level of J remaining in the JBP1 (–/–) (5%) is due to JBP2.

Utilizing the TMF assay, we show that in the absence of JBP2 newly generated telomeres located in the active 221 expression site lack base J. It has previously been shown that in wild-type cells the 50 bp repeat array immediately upstream of the (active) expression site (ES), contains significant levels of base J [5]. Therefore, in the absence of JBP2, JBP1 is unable to propagate J from the 50 bp repeats into the newly generated telomeric arrays approximately 50 kbp downstream. This apparent block in JBP1 stimulated J-propagation within the active 221 ES may be due to distance constraints or the effect of on-going transcription on the propagation mechanism. Based on the analysis of the JBP1/J-DNA interaction, we have previously proposed that the high rate of polymerase I transcription explains the exclusive lack of J in the active expression site [13]. The ability of JBP1 to bind J-DNA is essential for propagation [11] (Sabatini unpublished data). Also, JBP1 does not bind single-stranded J-DNA or the RNA/J-DNA duplex [13]. Thus, the high rate of polymerase I transcription could inhibit JBP1 stimulated J-propagation within the active expression site polycistronic transcription unit. Our *in vivo* analysis of J synthesis in newly generated telomeric arrays in the JBP2 (–/–) is consistent with this conclusion.

It is now clear, from the results presented here and earlier studies, that in bloodstream form trypanosomes JBP2 initiates de-novo J-synthesis while JBP1 maintains and propagates ongoing synthesis. The maintenance of high levels of J in the genome of the JBP2 (–/–) trypanosome, as shown here, is presumably due to the action of JBP1. Therefore, according to our model for the regulation of J-biosynthesis, subsequent deletion of JBP1 from the JBP2 (–/–) cell line should result in the complete lack of base J in the genome of bloodstream form trypanosomes. Recent results confirm this hypothesis (Cliffe, Kieft and Sabatini, manuscript in preparation). The analysis of this base J null mutant will allow the direct determination of the biological function of base J.

Materials and Methods

Enzymes and Chemicals

PMSF and Protease inhibitor tablets (Cocktail + EDTA) were from Roche, the thiol protease inhibitor FMK 024 (morpholinourea-phenylalanine-homophenylalanine-fluoromethyl ketone) was from MP Biomedicals; all other protease inhibitors were from Sigma-Aldrich. Benchmark Pre-stained protein standard, 1 KB plus DNA ladder and pCR 2.1 vector were purchased from Invitrogen, the 2.5 kb Molecular Ruler was purchased from Bio-Rad. Prime-It II random primer labeling kit was purchased from Stratagene, α -³²P dATP was purchased

from Perkin Elmer. ECL western blotting detection reagents and Hybond-N+ were from Amersham. All other enzymes were purchased from New England Biolabs.

Trypanosome Growth and Transfection

Bloodstream form *T. brucei* cell line 221a of strain 427 were cultured as described previously [11]. Bloodstream form transfections have been previously described [12]. The pTMF construct was a gift from David Horn, the JBP2 KO constructs (pTBT-JBP2, pTHT-JBP2) were gifts from Piet Borst.

Generation and analysis of *T. brucei* transfectants

The constructs used to generate *T. brucei* JBP2 KO mutants contain either a hygromycin phosphotransferase (*Hyg*) gene, or a blasticidin S deaminase (*BSR*) gene in between 240 bp of β - α tubulin and 330 bp of α - β tubulin intergenic sequences. A 0.76 kbp 5' (HindIII - NarI) and a 3.1 kbp 3' (NarI - HindIII) JBP2 flanking sequence were used as targeting fragments, replacing nts -784 to +3377 relative to the translation start site. 2.5 micrograms of either knockout construct digested with NotI and ApaI was used to transform WT *T. brucei* 221a. Transformants were selected for resistance to 2 μ g of blasticidin per ml or 2.5 μ g of hygromycin per ml. Two rounds of transformation were used to delete both JBP2 alleles. Correct integration of the resistance cassette into both JBP2 loci was verified by Southern blot analysis. Briefly, genomic DNA from wild type and mutant cell lines was digested with EcoRV, separated on a 0.5% agarose gel and transferred onto a Hybond N+ membrane. Filters were hybridized with a JBP2 upstream and ORF probe, PCR amplified with a JBP2 sp (5'-CGCCTCCCATATACGACGCGG-3') and a JBP2 asp (5'-CGTCCACGCGCTGTCCGAGAG-3'). Filters were washed to a final stringency of 0.1 X SSC, 0.1% SDS at 63°C. As expected an additional 20 kbp EcoRV fragment is present upon correct integration of the construct, coinciding with a loss of the WT fragment. Total loss of JBP2 ORF sequences was verified by hybridization with a full-length ORF probe (data not shown).

As an independent means of generating the JBP2 KO, we subsequently deleted the second JBP2 allele by loss of heterozygosity. To do this we grew the JBP2 $-/+$ hygromycin single allele KO in increased hygromycin drug pressure (30 μ g per ml) for three days. (The IC₅₀ of the JBP2 $-/+$ cell line was previously determined to be in the order of 20–25 μ g per ml) Following the initial increase in drug pressure the cells were grown in the absence of drug for one passage, after which they went through a second round of high selection (30 μ g per ml). Cells that grew out during this second round of selection were cloned out by limiting dilution and analyzed by PCR for the loss of the remaining JBP2 allele. DNA was isolated and analyzed for levels of base J in the genome. In all clones analyzed, JBP2 KO cell lines generated by LOH and conventional double transformation indicate an identical 5 fold reduction in J levels (data not shown).

Five microgram of the pTMF construct was digested with SmaI and used to transform WT *T. brucei* 221a and the JBP2 ($-/-$) trypanosomes. Transformants were selected for resistance to 2 μ g of geneticin per ml. Correct integration of the construct into the 221 ES was verified by Southern blot analysis. Briefly, genomic DNA from WT transfectants and JBP2 ($-/-$)

transfectants was digested with XhoI, separated on a 0.4 % agarose gel (overlayed on a 1 % agarose gel) and transferred onto a Hybond N+ membrane. Filters were hybridized with a neomycin phosphotransferase (NEO) ORF probe, PCR amplified with a NEO sp (5'-ACAAGATGGATTGCACGCAGG-3') and a NEO asp (5'-CAGAAGAAGTCTCGTCAAGAAGAAGG-3'). Filters were washed to a final stringency of 0.1X SSC, 0.1 % SDS at 63 °C and subjected to autoradiography. Filters were then stripped 4 times with a boiling solution of 0.1% (w/v) SDS and re-hybridized with a VSG 221 ORF probe (PCR amplified with a VSG 221 sp (5'-CTCGCCGTCTTGGTCGTAGCCC-3') and a VSG 221 asp (5'-AAAGAGGGGTCTTGCTAATGA-3'), TA cloned in the pCR2.1 vector. The probe fragment was released by digestion with EcoRI. Filters were washed to a final stringency of 0.1X SSC, 0.1 % SDS at 63 °C and subjected to autoradiography.

pGFP-JBP2 plasmid was digested with NotI and XhoI and used to transform the JBP2 (–/–) cells as previously described [11].

Generation of anti-JBP2 antisera and western blotting

JBP2 antisera were generated from synthetic peptides by Alpha Diagnostic International. Rabbits were immunized with synthetic peptides conjugated to KLH corresponding to the last 20 amino acids from the C-terminus of the SWI2/SNF2 subunit of JBP2 (1058 PAVTKIIRNIDTVKSERWI 1077). For protein lysates, cells were resuspended to a density of 10⁹/ml in ice-cold Hepes-NaCl Buffer (21 mM Hepes pH 7.0, 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, 6 mM Glucose) supplemented with protease inhibitors (see below), then resuspended 1:1 in lysis buffer supplemented with protease inhibitors (final concentrations are: 50 mM Tris pH 6.8, 2 % SDS, 10 % Glycerol, 0.05 % Bromophenolblue, 100 mM DTT, 2 mM Iodoacetamide, 0.08 mM FMK 024, 0.4 mM PMSF, 0.02 mM TLCK, 40 ug/ml Aprotinin, 8 ug/ml Leupeptin, 8 ug/ml Pepstatin and a 2-fold higher concentration as recommended of the protease inhibitor cocktail). The lysates were boiled at 94 °C for 5 min. Proteins from 8 x 10⁶ cell equivalents were separated on a 6 % SDS-PAGE gel, transferred onto nitrocellulose, and probed with anti-JBP2 antisera (second boost, diluted 1:4000). Bound antibodies were detected by a secondary goat anti-rabbit antibody, conjugated to HRP and visualized by ECL.

Determination of the genomic level of J

To quantify the genomic J levels, we used the anti-J DNA immunoblot assay [5]. Briefly, serially diluted genomic DNA was blotted to nitrocellulose and incubated with anti-J antisera. Bound antibodies were detected by a secondary goat anti-rabbit antibody, conjugated to HRP and visualized by ECL. The membrane was stripped and hybridized with a probe for the β -Tubulin gene to correct for DNA loading.

Total genomic DNA was isolated as described [14] and resuspended in 10mM Tris-HCL/1mM EDTA (pH 7.4).

Determination of the sequence distribution of J

This was done as previously described [11].

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Figure 1. JBP2 is involved in J-Biosynthesis in Bloodstream Form Trypanosomes

A. Restriction enzyme map of the JBP2 gene locus, with the constructs used to disrupt JBP2 expression shown below. In each case, the JBP2 ORF is replaced by an expression cassette giving either Blasticidin (BSR) or hygromycin (Hyg) resistance. All ORF's are indicated with grey boxes. Both resistance genes are flanked by α -tubulin intergenic regions (hatched boxes) for proper processing. Restriction enzyme sites used for making the constructs and for mapping of the transformants are indicated: EcoRV (E), HindIII (H), NarI (N). The probe fragment is shown above the map. The 3.6 kbp WT and 20 kbp KO hybridizing EcoRV fragments are indicated (dotted line).

B. Southern mapping of bloodstream form *T. brucei* cell line 221a of strain 427 transformed with the JBP2 knockout constructs. Genomic DNA from wildtype cells (WT), heterozygous (JBP2 +/- (BSR)) and homozygous (JBP2 -/- (BSR/Hyg)) JBP2 knockout cells was digested with EcoRV and hybridized with a JBP2 upstream and ORF probe. Size markers are indicated in kbp.

C. JBP2 is absent in JBP2 -/- bloodstream form trypanosomes. Total cell extracts of the following cell lines were analyzed by Western blotting using anti-JBP2 antisera (α -JBP2): Procyclic 29-13 cells (PC WT), Procyclic WT cells + GFP-JBP2 (PC WT + JBP2), Bloodstream form 221a WT cells (BS WT) and Bloodstream form JBP2 -/- cells (BS JBP2 -/-). The 120 kDa wild type JBP2 protein and the 150 kDa GFP-JBP2 fusion protein are indicated, as well as the position of the size markers (in kDa). The blot was subsequently hybridized with anti-La antisera (α -La) to control for protein loading.

D. Specificity of the JBP2 antisera. Total cell extract from procyclic cells expressing the GFP-JBP2 fusion was analyzed by western blot using pre-immune serum (PIM) or anti-JBP2 antisera (Anti-JBP2) at the same dilution.

E. J levels in the DNA of JBP2 knockout cells. The J content was determined with anti-J DNA immunoblots. Dot blots with twofold dilution series of DNA from Bloodstream form 221a WT cells (WT), heterozygous JBP2 knockout cells (JBP2 +/- (BSR)), homozygous JBP2 knockout cells (JBP2 -/- (BSR/Hyg)) and homozygous JBP2 knockout cells (JBP2 -/- (BSR/Hyg)) with the ectopically expressed JBP2-GFP were incubated with anti-J antisera. Procyclic (PC) DNA was included as a negative control. Bound antibodies were detected by a second antibody conjugated to horseradish peroxidase and visualized by ECL (left panel). DNA loading was checked by hybridization with a probe specific for the β -Tubulin gene (right panel).

F. Localization of J in the JBP2 knockout cell line. DNA from the indicated cell lines was sonicated and immunoprecipitated with anti-J antisera and blotted onto nitrocellulose. The blots were then hybridized with a radiolabeled DNA probe corresponding to the indicated regions of the genome.

Figure 2. JBP2 Stimulates *De Novo* J-Synthesis

A. Generation of new telomeric arrays in the 221 VSG expression site by Telomere Fragmentation. As described previously [12], transfection of *T. brucei* with pTMF replaces the native telomere at the 221 bloodstream form expression site (ES) with a new telomere seed 5 kbp downstream of the ribosomal RNA promoter (P_{rRNA}). The question mark refers to the unknown presence of J within newly generated telomeric arrays, both in WT and in JBP2^{-/-} cells transfected with the pTMF construct. The plasmid was digested with SmaI (M) prior to transfection. Restriction enzyme sites used for mapping of the transformants are indicated: XhoI (X), Bsp120I (B) and ScaI (S). The probe fragments are shown underneath the map.

B. Growth of newly generated telomeres in WT and JBP2^{-/-} cells. From WT cells DNA was isolated 15 and 40 days post transfection; from JBP2^{-/-} cells 18 and 60 days post transfection. DNA was digested with Xho I and analyzed by Southern blotting. Similar hybridization patterns with the NEO and 221 VSG probe confirm proper integration of the construct in the 221 ES

C. JBP2 stimulates *de novo* J-synthesis in newly generated telomeric DNA. All indicated cell lines were transfected with the pTMF construct. Clones were obtained after which

genomic DNA from transfected wildtype cells (WT), transfected homozygous JBP2 knockout cells (JBP2 $-/-$), transfected homozygous JBP2 knockout cells (JBP2 $-/-$) grown in the presence of 1 mM HOMedU (HMU) and transfected homozygous JBP2 knockout cells (JBP2 $-/-$) with the ectopically expressed JBP2-GFP, was isolated and digested with XhoI. An immunoprecipitation was performed with anti-J antisera, after which 10% of the input (In) DNA and 100% of the immunoprecipitated (IP) DNA was analyzed by hybridization with a NEO probe.

D. IP efficiency in the JBP2 $-/-$. DNA was isolated from TMF transfected wild type (WT) and JBP2 knockout (J2KO) and analyzed by immunoprecipitation as described in C. The DNA samples were then dot-blotted onto nitrocellulose and hybridized with the NEO probe (left) and 177bp probe (right).

E. J levels in the DNA of JBP2 knockout cells. The qualitative assessment of J content of the cell lines described in panel C was determined with anti-J DNA immunoblots and ECL detection, as described in Fig. 1 E.