Regulation of Trypanosome DNA Glycosylation by a SWI2/SNF2-like Protein

Courtney DiPaolo,1 Rudo Kieft,1 Mike Cross,2 and Robert Sabatini1,*
1Global Infectious Diseases Program
Marine Biological Laboratory
Woods Hole, Massachusetts 02543
2Division of Molecular Biology and
The Centre of Biomedical Genetics
The Netherlands Cancer Institute
1066 CX Amsterdam
The Netherlands

Summary

Synthesis of the modified thymine base β-D-glucosylhydroxymethyluracil, 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. The mechanism of developmental and telomeric-specific regulation of J synthesis is unknown. We have previously identified a J binding protein (JBP1) involved in propagating J synthesis. We have now identified a homolog of JBP1, JBP2, containing a domain related to the SWI2/SNF2 family of chromatin remodeling proteins that is upregulated in bloodstream form cells and interacts with nuclear chromatin. We show that expression of JBP2 in procyclic form cells leads to de novo J synthesis within telomeric regions of the chromosome and that this activity is inhibited after mutagenesis of conserved residues critical for SWI2/SNF2 function. We propose a model in which chromatin remodeling by JBP2 regulates the initial sites of J synthesis within bloodstream form trypanosome DNA, with further propagation and maintenance of J by JBP1.

Introduction

In the DNA of kinetoplastid flagellates, a fraction of thymine is replaced by the modified base β-D-glucosylhydroxymethyluracil, called J (Gommers-Ampt et al., 1993; Borst and van Leeuwen, 1997; van Leeuwen et al., 1998b). This hypermodified 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. Although J is abundantly present in telomeric repeats of all kinetoplastids, it is also found in the subtelomeric variant surface glycoprotein (VSG) gene expression sites involved in antigenic variation in the parasite T. brucei (van Leeuwen et al., 1996, 1997, 1998b). By periodically switching its VSG coat, the trypanosome is able to evade the host immune system in chronic infections. The VSG that is expressed and localized on the surface of the cell is encoded by a gene transcribed from one of about 20 telomeric VSG expression sites. Only one of the 20 expression sites is active at a 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 (van Leeuwen et al., 1997, 1998a, 1998b).

In trypanosomes J might therefore fulfill the role played in mammals by methylcytosine (MeC) (Jones et al., 1998), a base not present in T. brucei (Gommers-Ampt et al., 1993). 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 life stage) and not during the procyclic life stage that is characterized by infection of the midgut of the vector, the tsetse fly. Although much is known about the localization of J, little is understood concerning the regulation of J biosynthesis. Substantial indirect evidence (Borst et al., 1993; van Leeuwen et al., 1998a; Ulbert et al., 2002) indicates that the modification of thymine in trypanosome DNA occurs in two steps: a thymine is oxidized to hydroxymethyluracil (HOMeU) by a putative thymine-7-hydroxylase (TH), followed by glucosylation of the new base by a putative glucosyltransferase (GT). This GT is present in both trypanosome life stages and can glucosylate HOMeU anywhere in nuclear DNA, as shown by the global presence of J in the DNA of trypanosomes grown in the presence of the J precursor, 5-hydroxymethyldeoxyuridine (HOMeD) (van Leeuwen et al., 1998a). In contrast, it has been hypothesized that the putative TH can only oxidize thymines within limited regions of the chromosome, somehow marked by chromatin structure or intranuclear location. The potential bloodstream trypanosome-specific expression of TH would therefore regulate both the site-specific and developmental regulation of J modification. The gene for either of the two enzymes involved in J synthesis has not been identified.

The search for proteins involved in J function led to the isolation of a protein from trypanosome nuclear extracts that is able to bind base J in DNA, called the J binding protein or JBP1 (Cross et al., 1999). JBP1 binds J DNA in a manner similar to the Myb-like homeodomain family of duplex telomere binding proteins (Sabatini et al., 2002a, 2002b). Consistent with this idea, JBP1 contains a Myb-like DNA binding motif (Cross et al., 1999). Analysis of the bloodstream form, null mutant trypanosome has indicated that JBP1 represents a propagation and inheritance factor for J biosynthesis (Cross et al., 2002). JBP1 presumably binds J DNA and activates (i.e., recruits) the synthetic 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 after replication (inheritance). As such, JBP1 represents the first cofactor involved in J biosynthesis that has been identified.

In this study, we demonstrate that JBP1 can function as a maintenance factor for J synthesis when expressed in procyclic trypanosomes and provided a “seed” of J in the genome. Therefore, both trypanosome developmental life stages contain a fully functional J synthesis...
pathway, including TH activity. We describe a homolog of JBP1, JBP2, that contains a region homologous to the family of SWI2/SNF2-like chromatin-remodeling proteins. We show that JBP2 expression in trypanosomes stimulates de novo site-specific synthesis of a basal level of J along the chromosome, which can be further propagated and maintained by JBP1. JBP2 is developmentally regulated and interacts with nuclear chromatin, and mutations within the SWI2/SNF2 domain inhibit the stimulation of J biosynthesis. Based on these studies we propose a model where chromatin remodeling by JBP2 represents the critical process for regulating J biosynthesis in bloodstream form trypanosome DNA.

Results

JBP2 Represents a SWI2/SNF-like Protein

During the search for additional J binding proteins, we identified a JBP1 homolog in the T. brucei genome database (accession number Tb07.26A24.600). This 2.5 kb ORF corresponds to a theoretical protein of 120 kDa that contains an N terminus with homology to JBP1 (34% identity, 47% similarity) including the putative Myb DNA binding motif (Cross et al., 1999) (Figure 1A). Because of the homology to JBP1, we refer to this protein as JBP2. The remaining C-terminal half of JBP2 has homology (24% identity, 45% similarity) to the SWI2/SNF2 family of ATPase DNA helicase proteins involved in chromatin remodeling. A similar homolog also exists in the Leishmania major genome database (accession number AL512295.2). As shown in Figure 1, JBP2 contains a domain homologous to representatives of the SWI2/SNF2 subfamily of proteins from yeast, humans, Drosophila, and flowering plants. This region contains the characteristic seven conserved motifs that are present in the large group of nucleotide triphosphate (NTP) binding proteins that include DNA helicases. Motif IV has the lowest similarity (Figure 1B). Helicase motifs I and II are involved in ATP binding and hydrolysis, respectively. The function of the remaining motifs is unclear. Additional sequence elements that are highly conserved specifically among SWI2/SNF2 family members are also shown and denoted IVb and Vb. The homology between JBP2 and SWI2/SNF2 also includes specific residues that have been implicated in ATP hydrolysis and SWI2/SNF2 function (Richmond and Peterson 1996, and references therein). We conclude that JBP2 represents a SWI2/SNF2-like protein that contains an N-terminal domain with homology to J binding protein 1 (JBP1).

JBP2 Is Developmentally Regulated and Localized to the Nucleus in T. brucei

To examine the regulated expression of JBP2 we performed Northern blot analysis using RNA from bloodstream and procyclic life stages. Using a large quantity of poly A RNA, we were able to detect the specific expression of full-length JBP1 mRNA in bloodstream verses procyclic form trypanosomes, but unable to detect a signal corresponding to JBP2 mRNA in either life stage (data not shown). Therefore, as a more sensitive approach, we performed reverse transcriptase-polymerase chain reaction (RT-PCR). The RT-PCR analysis using a JBP1-specific primer indicated the presence of JBP1-specific transcripts in bloodstream form and significantly less in procyclic form corresponding to an approximate 10-fold downregulation of JBP1 mRNA (Figure 2A). However, analysis of the calmodulin control
Figure 2. JBP1 and JBP2 Are Developmentally Regulated and Localized to the Nucleus in *T. brucei*

(A) JBP1 and JBP2 expression analysis by RT-PCR. RT-PCRs were performed on RNA from procyclic (PC) and bloodstream (BS) cells as described in Experimental Procedures. Samples were taken after the indicated number of cycles, run out on agarose gels, and stained with ethidium bromide (EtBr), after which they were transferred and hybridized with the indicated probes (Hyb). As a negative control, a –RT reaction was taken for the indicated number of cycles. For the control calmodulin RT-PCR the relative amounts of hybridization between PC and BS are indicated below.

(B) Western blot analysis of JBP-GFP expression. Procyclic cell lysates, from cells expressing JBP1-GFP and JBP2-GFP, in SDS-PAGE sample buffer were examined by Western blot analysis with anti-GFP.

(C) Intracellular localization of JBP1-GFP and JBP2-GFP in procyclic trypanosomes. Live, DAPI-stained trypanosomes on agarose slides were visualized by microscopy. DAPI staining reveals the localization of the nucleus (N) and kinetoplast (mitochondrial DNA; K).

RT-PCR samples (10–18 PCR cycles) suggest that the level of steady-state JBP1 mRNA, even in the bloodstream form trypanosome, is low. Analysis of the JBP2 RT-PCR titration reactions (Figure 2A) indicates that JBP2 expression at the steady-state RNA level is also low, perhaps even significantly lower than JBP1, in bloodstream form trypanosomes. This may explain the difficulty detecting JBP2 mRNA by Northern blot analysis. Although there appears to be some level of mRNA expressed in procyclics, JBP2 is also clearly downregulated compared to levels in the bloodstream form trypanosome. Phosphorimager quantitation indicates this corresponds to approximately a 5-fold down regulation. The inability to express recombinant JBP2 has precluded any attempt to examine the JBP2 protein levels. Therefore, we predict that JBP2 and JBP1 are bloodstream form-specific genes expressed at extremely low levels.

To determine the cellular localization of JBP2, we expressed a GFP-fusion version (Figure 2B). As a positive
control we also expressed the JBP1-GFP fusion, since JBP1 was initially identified in trypanosome nuclear extracts (Cross et al., 1999). As shown in Figure 2C, JBP1 and JBP2 fused to the carboxy terminus of GFP were localized to the nucleus of stably transfected procyclic cells. The localization of JBP1 and JBP2 within the nucleus is not diffuse. Rather, they appear as high-intensity GFP signals that colocalize with 4,6-diamino-2-phenylindole (DAPI), suggesting chromatin association. It is unclear what the apparent punctate distribution of GFP-JBP localization represents. Since these are procyclic cells, the distribution of, for example, JBP1, should be independent of base J in the genome. In vitro analysis of the JBP1/J-DNA interaction indicates little if any binding of JBP1 to unmodified DNA (Sabatini et al., 2002a). There is no nuclear localization of GFP after expression of GFP lacking the JBP2- or JBP1- fused polypeptide (data not shown).

JBP2 Associates with Chromatin and Leads to J Synthesis via Functional ATPase Domain

Previously, we have demonstrated the ability of JBP1 to recognize J DNA using recombinant protein expressed in E. coli. To examine the affinity of JBP2 for J DNA using a similar in vitro gel-shift J-DNA binding assay, we attempted to produce recombinant protein without success. Therefore, we developed an in vivo J-DNA binding assay. As a positive control, we first examined JBP1 association with chromatin by detergent lysis of procyclic trypanosomes expressing JBP1-GFP fusion protein, followed by cell fractionation. As shown in Figure 3A, in these cells JBP1 is normally localized to the supernatant fraction along with the soluble nucleoplasmin-localized protein La (Marchetti et al., 2000). Procyclic trypanosomes lack base J unless they are fed HOMedU (van Leeuwen et al., 1998a). Therefore, the association of JBP1 with the “chromatin” fraction, identified by the presence of histone H3, is clearly dependent upon HOMedU (Figure 3A). The association of JBP1 with the chromatin fraction is stable up to 750 mM NaCl.

To examine whether JBP2 represents a J binding protein, we repeated the analysis using procyclic trypanosomes expressing the JBP2-GFP fusion. In contrast to JBP1, we see a fraction of JBP2 (30%–50%) associated with the chromatin fraction in the absence of HOMedU and no additional interaction following addition of HOMedU (Figure 3B). To control for nonspecific interactions, we show that >95% of the La protein is localized in the supernatant fraction. The ability to associate with chromatin in the absence of HOMedU and the lack of a response to HOMedU feeding would suggest that JBP2 is not a base-J-specific binding protein. However, when we analyzed the DNA from the JBP2-GFP cells we found base J, even in the absence of HOMedU (Figure 3C). Therefore, ectopic expression of JBP2 in procyclic trypanosomes leads to J synthesis. The level of J in the population of uncloned procyclic cells expressing JBP2 is only 2- to 4-fold less than the levels in the bloodstream JBP1 null mutant (Figure 3C). Increasing the expression of JBP2 via the tetracycline-regulatable T7 promoter system, does not lead to an increase in the levels of J or in the amount of association with chromatin (data not shown).

JBP2 contains several conserved residues that have been shown to be critical for SWI2/SNF2 function, including residues within helicase motifs I and II involved in ATP binding and hydrolysis, respectively (Figure 1). To examine the requirement of a functional ATPase motif for JBP2-stimulated J biosynthesis, we made alanine substitution mutations in the invariant lysine of motif I (K508A) and in the DE residues of motif II (DE657–658AA) of the SWI2/SNF2-like domain of JBP2 (Figure 3D). Each of these mutations has previously been shown to inhibit ATPase activity and SWI2/SNF2 function (Richmond and Peterson, 1996, and references therein). After transfection with the appropriate construct, we see that the procyclic trypanosomes express the mutant and wild-type JBP2-GFP fusion proteins to similar levels (Figure 3E), but only the wild-type JBP2 protein leads to J synthesis (Figure 3F). This would suggest that a functional SWI2/SNF2 ATPase motif is required for JBP2 stimulated J biosynthesis.

JBP2 and JBP1 Regulate Bloodstream Form-Specific J Biosynthesis

The ability of procyclic trypanosomes to synthesize J after incorporation of the intermediate HOMeUra in DNA, suggests they contain glucosyltransferase activity but normally lack J due to the absence of thymine hydroxylase activity. In order to directly address this, we tested whether JBP1 could maintain J synthesis when expressed in the procyclic cell. To do this we performed a pulse-chase experiment in which cells were grown in the presence of HOMedU for seven generations, washed, and allowed to continue growing in the absence of HOMedU. Loss of J was monitored by taking samples over time and quantifying the level of J by anti-J DNA dot-blot analysis. If this procedure is done using wild-type procyclic trypanosomes, we see the expected loss of J during the chase phase corresponding with simple dilution in multiplying cells (Figure 4A) (Blundell et al., 1998; Cross et al., 2002). However, if we repeat the pulse-chase procedure using a cell line allowing tetracycline-inducible expression of JBP1 (Figure 4C), we see that induction of JBP1 expression leads to the maintenance of J biosynthesis (Figure 4B). Therefore, although expression of JBP1 alone does not lead to de novo J synthesis (Figure 2 and 4B), it can support ongoing synthesis once a seed of J is provided. Probably due to the absence of JBP1 inducible expression, the retention of J can vary from 40%–80% of the initial levels after the HOMedU pulse. Similar to the activity of JBP1 in the bloodstream trypanosome (Cross et al., 2002) and based on mapping the blockage of restriction sites and Bal 31 digestion the JBP1-stimulated retention of J in procyclic cells seems to be global and not localized to any specific region of the genome (data not shown). The incomplete loss of J after the growth of these cells in the absence of tetracycline is potentially due to a small level of leaky expression of JBP1 from the construct.

Previous analysis of procyclic and bloodstream trypanosomes has indicated that J is not actively removed from DNA but is simply diluted out upon DNA replication (Blundell et al., 1998; Cross et al., 2002). Therefore, the retention of J in the procyclic cell following the HOMedU pulse represents JBP1-supported, ongoing J synthesis.
Chromatin Remodeling and Base J Synthesis

Figure 3. JBP2 Associates with Chromatin and Stimulates J Biosynthesis via a Functional ATPase Motif

(A) JBP1 associates with chromatin in a J-dependent manner. Procyclic trypanosomes transfected with pJBP1-GFP-tub-phleo and grown in the absence (−) and presence (+) of 1mM HOMedU were lysed and fractionated into supernatant (S) and pellet (P) as described in Experimental Procedures, run on an SDS-PAGE, blotted, and incubated with anti-GFP antisera to detect JBP1-GFP fusion protein. The same blot was subsequently analyzed with anti-La (La) and anti-histone H3 (H3) antisera.

(B) JBP2 associates with chromatin independent of HOMedU-stimulated J synthesis. Procyclic cells expressing JBP2-GFP were analyzed by cell fractionation as in (A).

(C) JBP2 stimulates J biosynthesis. DNA was isolated from the indicated trypanosome cell lines and analyzed for J content by spotting DNA diluted in 1:1 steps onto a membrane that was then incubated with J-antisera. wt, wild-type cells; J1KO, bloodstream JBP1 null; J1, procyclic cells expressing JBP1-GFP; J2, procyclic cells expressing JBP2-GFP.

(D) Schematic diagram of JBP2 indicating alanine substitution mutations within ATPase motifs I and II. Underlined residue(s) indicate those substituted with alanine to produce the JBP2-GFP mutant fusion proteins K550A and DE657-658AA.

(E) Expression of wild-type and mutant JBP2. Procyclic trypanosomes expressing wild-type JBP2 (WT) and the indicated JBP2 mutants (550, K550A; 657-658, DE657-658AA) were analyzed by Western blot using anti-GFP antisera. Extract from untransfected procyclic cells (C) is included as a negative control. The blot was subsequently hybridized with anti-La (La) to control for protein loading.

(F) JBP2 ATPase mutants are unable to support J biosynthesis. DNA was isolated from the indicated cell lines and analyzed for J content as described in (C).

and not protection from J removal. This would suggest that procyclic cells do contain thymine hydroxylase activity and that JBP1 function requires the presence of a small amount of J in the genome on which to bind to stimulate further J biosynthesis.

In the bloodstream trypanosome, the limited amount of J essential for JBP1-stimulated J biosynthesis may be provided by JBP2. To examine this possibility, we determined whether JBP1 can function in the procyclic trypanosome via J provided by JBP2 expression by co-transfecting the procyclic 29-13 cell line with the JBP2-GFP-tub-phleo and JBP1-HA-pHD615 construct. The resulting cell line, J1/J2, allows constitutive expression JBP2-GFP and tetracycline-regulated expression of JBP1-HA (Figure 5A). If we examine the J DNA content of these cells, in the absence of tetracycline we see the basal level of J synthesis due to JBP2 expression, which can then be propagated (5-fold) upon induction of JBP1 expression by tetracycline (Figure 5B).

The lack of even a basal level of J in the genome of wild-type procyclic trypanosomes and their inability to maintain J after HOMedU incorporation unless JBP2 or JBP1 is ectopically expressed, respectively, is consistent with the developmental regulation of JBP2 and JBP1. These results, including the ability of JBP1 to propagate the basal level of J provided by JBP2, also suggest that JBP2 represents a critical factor in the regulation of J biosynthesis in bloodstream form trypanosome DNA. We conclude that the procyclic trypanosome contains a full complement of J biosynthesis enzymes, and JBP2 and JBP1 represent important regulators of the life-stage-specific synthesis of base J.

JBP2 Regulates the Genomic Distribution of Base J

To examine the chromosomal distribution of J due to the expression of JBP2 and JBP1, genomic DNA was sheared by sonication and then analyzed by immunoprecipitation using J antisera (van Leeuwen et al., 1997). Immunoprecipitated fragments were identified by dot-blot hybridization (Figure 6A). The amount of anti-J immunoprecipitation reflects the density of modification...
Figure 4. Procyclic Cells Contain Thymine-Hydroxylase Activity

(A) J is normally diluted out of replicating chromatin. The 29-13 procyclic cell line was grown in the presence of 1 mM HOMedU (HMU) for several generations (pulse) followed by a 6 day chase in the absence of HOMedU. J content of chromosomal DNA was analyzed for each sample indicated, as described in Figure 3.

(B) JBP1 can maintain J synthesis in procyclic cells. A similar HOMedU pulse-chase experiment was done using the 29-13 cell line transfected with JBP1-HA-pHD615. During the 6 day chase in the absence of HOMedU, cells were grown in the absence (−) or presence (+) of tetracycline (Tet) and DNA isolated, as indicated. J content of each DNA sample was analyzed as in (A).

(C) Tetracycline-regulated expression of JBP1. Total cell extract from the transfected cell line in B grown in the absence or presence of tetracycline was analyzed by Western blot. JBP1 expression was detected using anti-HA antisera. Anti-histone H3 (H3) was provided as a control for protein loading.

in a semiquantitative manner (van Leeuwen et al., 1997). From the DNA of the J1/J2 cell line induced with tetracycline and expressing both JBP2 and JBP1, regions of the chromosome that are known to contain J in wild-type bloodstream trypanosome were immunoprecipitated (i.e., an IP efficiency of 11.2% for the 177 bp region). On the other hand, tubulin, enolase, calmodulin, and actin were not precipitated (average IP efficiency of 0.2%), indicating a lack of J in these regions (Figure 6A) (data not shown). A similar immunoprecipitation profile is present using DNA from the bloodstream trypanosome. From the DNA of procyclic cells grown in the presence of HOMedU, all sequences tested were immunoprecipitated, showing that J was present throughout the genome (van Leeuwen et al., 1998a). Also, we saw no significant precipitation of any sequence tested using wild-type procyclic DNA that acted as a negative control. Therefore, the concomitant expression of JBP2 and JBP1 in the procyclic cell is able to closely mimic the localization of J seen in wild-type bloodstream trypanosome DNA.

To address the contributions of JBP2 verses JBP1 to the specificity and levels of J synthesis in these cells, we compared the localization of J in the DNA of the J1/J2 procyclic trypanosome grown in the absence and presence of tetracycline. In the absence of tetracycline, and therefore the absence of JBP1 expression, we see similar specific localization of J along the chromosome as described above for the JBP1/JBP2 double expressor (Figure 6B and data not shown). Upon induction of JBP1 synthesis, propagation of additional J synthesis is approximately proportional to the basal level of J introduced within each specific region by JBP2. Therefore, we conclude that JBP2 leads to site-specific synthesis of J in procyclic trypanosomes within regions of chromosome that normally contain J in the bloodstream chromosome. JBP1 is then able to act via this basal level of J and propagate further synthesis within these localized regions of the chromosome.

Discussion

*T. brucei* and related kinetoplastid flagellates contain the modified base J in their telomeric DNA. In *T. brucei*, J is developmentally regulated and abundant in repetitive sequences present at subtelomeric expression sites involved in the regulation of antigenic variation. Whereas the amount and localization of J is known in some detail, little is known about its synthesis or function. Nucleoside-feeding studies have led to the model of J synthesis involving the intermediate HOMedU and the two enzymes thymine-hydroxylase and glucosyltransferase.
Figure 6. Specific Localization of J After JBP2 and JBP1 Expression
(A) Localization of J within bloodstream-specific sites along the chromosome. DNA was extracted from the indicated cell lines, sonicated, and fragments containing J were immunoprecipitated using the J-antisera. 10% of the input DNA (Input) and 100% of the immunoprecipitate (IP) were blotted onto nitrocellulose. Blots were then hybridized with radiolabeled DNA probes corresponding to indicated regions of the trypanosome chromosome. Representative autoradiographs of each blot from three to five separate experiments are shown. WT represents wild-type procyclic (PC) or bloodstream (BS) cells as indicated. H11001 represents procyclic cells grown in the presence of 1 mM HOMedU. The unequal distribution of J within the chromosome after HMU feeding of procyclic trypanosomes (i.e., differential stimulation in IP efficiency of the 50 bp region) has previously been shown (van Leeuwen et al., 1998a).

(B) JBP1-stimulated propagation. J immunoprecipitations from the J1/J2 cell line grown in the absence (−) versus presence (+) of tetracycline were done in triplicate as above and quantitated and expressed as %IP as previously described (van Leeuwen et al., 1998a; Cross et al., 2002). The data are normalized against any background precipitation corresponding to each region from wt procyclic DNA. 5S is an internal region of the chromosome that has previously been shown to contain low levels of base J (van Leeuwen et al., 2000; Cross et al., 2002).

(Figure 7). This two-step reaction scheme was recently supported by the finding that the intermediate used for J synthesis, HOMedU, is freely accessible to enzymatic removal from trypanosome DNA by a human DNA glycosylase, hSMUG1 (Ulbert et al., 2002). The ability of procyclic cells to convert HOMedU to J but still normally lack J in the genome, has suggested that the developmental regulation and chromosomal localization of J synthesis are regulated by the thymine-hydroxylase. We show here that procyclic cells contain a functional J synthesis machinery, including thymine-hydroxylase, and identify JBP1 and JBP2 as key regulatory cofactors of J biosynthesis. We propose a model for the regulation of J biosynthesis involving chromatin remodeling by a SWI2/SNF2-like protein, JBP2.

JBP1 was initially isolated from kinetoplastid nuclear extracts as a factor that specifically recognizes J DNA (Cross et al., 1999). Subsequently, we have further characterized the ability of recombinant JBP1 protein to specifically recognize and bind double-stranded J DNA via a similar domain to the Myb-helix-turn-helix-type DNA binding protein family (Sabatini et al., 2002a, 2002b). This type of binding domain is utilized in all duplex telomere binding proteins identified thus far. Gene knockout of JBP1 (Cross et al., 2002) and ablation of JBP1 mRNA by RNAi (R.S., unpublished data) resulted in a 20-fold reduction in J levels in bloodstream form trypanosomes. Based on these studies, we proposed that JBP1 represents a propagation and inheritance factor for J synthesis (Cross et al., 2002). Presumably JBP1 binds residual levels of J and directly or indirectly stimulates further synthesis along the DNA (propagation) as well as on the opposing strand after replication (inheritance). The ability of JBP1 to specifically bind J DNA would be essential for this activity. We illustrate here that association of JBP1 with chromatin in vivo is dependent upon the presence of J. Furthermore, mutations in the putative Myb-like domain of JBP1 inhibit J-DNA binding and the propagation of J synthesis in vivo (C.D. and R.S., unpublished data). Therefore, JBP1 does represent a J binding protein in vivo, and this interaction is crucial for its ability to propagate J synthesis along the chromosome. We show here that JBP1 is developmentally regulated, and ectopic expression in procyclic cells does not lead to J synthesis. However, if we provide a seed of J in the procyclic genome either by feeding the cells HOMedU or by expression of JBP2, JBP1 can propagate and maintain J synthesis. These results not only support the identification of JBP1 as a propagation/inheritance factor for J synthesis but also suggest that procyclic cells contain thymine-hydroxylase as well as glycosyltransferase activity. Therefore, the lack of base J in the procyclic genome has to be explained by some other regulated factor.

We propose that JBP2 represents such a factor. JBP2 is a developmentally regulated gene that when ectopi-
Molecular Cell

Figure 7. JBP2 Regulation of J Biosynthesis

A model for the developmental regulation of J biosynthesis indicating proposed roles of JBP2 as a chromatin remodeling protein and JBP1 as a propagation factor is presented as discussed in the text. According to the model, JBP2 binds specific regions of the chromosome (i.e., repetitive DNA, indicated by chromatin in bold) and catalyzes the remodeling of chromatin allowing access of the thymine-hydroxylase to a thymine base in DNA that, after glycosyltransferase activity, results in its conversion to J. Subsequent binding of JBP1 to J modified DNA allows additional access of the synthetic machinery to adjacent thymine residues, presumably via direct recruitment of the thymine-hydroxylase, resulting in the spreading (propagation) of J along the chromosome. Arrows in red represent developmentally regulated processes (i.e., bloodstream specific). Therefore, the lack of base J in procyclic trypanosome DNA is primarily due to the lack of JBP2 and the inability of the thymine-hydroxylase to effectively access thymine residues in chromatin.

Abbreviations: TH, thymine-hydroxylase; GT, glycosyltransferase; and Glc, glucose.

cally expressed in procyclic cells leads to the site-specific synthesis of base J. The lack of base J in the genome of wild-type procyclic cells is consistent with the regulated expression of JBP2 and the corresponding lack of effective protein in these cells. Therefore, the ability of JBP1 to support the maintenance of a high-level of base J synthesis in procyclic trypanosomes would suggest that these cells have sufficient levels of thymine-hydroxylase in the absence of JBP2. The presence of significant thymine-hydroxylase activity in the absence of JBP2 expression and the lack of detectable J unless JBP2 is ectopically expressed strongly argues against the possibility of JBP2 representing the thymine-hydroxylase. Microscopy and biochemical analysis of procyclic cells expressing the JBP2-GFP fusion protein suggest that at least a fraction of JBP2 has the ability to interact with chromatin in vivo. Whether the potential interaction of JBP2 with the trypanosome chromosome is directly linked with its ability to localize J biosynthesis is unknown. However, based on the data presented here, we propose that JBP2 interacts with chromatin and stimulates the ability of the thymine-hydroxylase to convert specific thymines to HOMedU.

It is becoming clear that chromatin remodeling may represent a conserved mechanism of maintaining methylation patterns in genomes of mammals and flowering plants. SWI/SNF chromatin-remodeling complexes regulate gene expression by disrupting histone-DNA interactions and permitting a variety of proteins to access DNA (Muchardt and Yaniv, 1999; Fiaus and Owen-Hughes, 2001; Langst and Becker, 2001). Central to this activity are SWI2/SNF2-type ATPases that form the catalytic core of these remodeling complexes. The SWI2/SNF2 protein family falls within the large superfamily of DEXD/H-ATPases that participate in various nuclear activities including transcriptional control (Sudarsanam et al., 2000; Fazzio et al., 2001), DNA repair (Eisen et al., 1995), chromosome segregation (Yoo et al., 2000), and chromosome folding (Deuring et al., 2000; Yoo et al., 2000). Recently, several members of the SWI2/SNF2-type subfamily have been shown to be involved in the control of DNA methylation status. Lymphoid specific helicase (Lsh) has been shown to regulate DNA methylation levels in repetitive sequences in mice (Dennis et al., 2001). Mutations in another member of the SWI2/SNF2 family, ATRX, give rise to changes in the pattern of methylation of several repeated sequences including the rDNA arrays, a Y specific satellite, and subtelomeric repeats (Gibbons et al., 2000). In the plant Arabidopsis thaliana, loss of DDM1 (deficient in DNA methylation1) function causes a 70% reduction of genomic cytosine methylation, with most of the loss occurring in repetitive sequences (Kakutani et al., 1995). DDM1 has been shown to have ATPase activity and possesses the ability to bind nucleosomes and promote nucleosome repositioning in an ATP-dependent manner (Brzeski and Jerzmanowski, 2003). Therefore, it is speculated that these SWI2/SNF2-like proteins maintain DNA methylation status by remodeling chromatin structure to facilitate the access of DNA methylases to the substrate (Jeddeloh et al., 1999).

Although JBP2 was initially identified based on 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 two additional sequence elements that are highly conserved only in members of the SWI2/SNF2 subfamily (Gorbalevaya et al., 1989; Eisen et al., 1995; Richmond and Peterson, 1996). The ability of JBP2 to localize J synthesis in the procyclic genome is inhibited after mutation of residues implicated in ATPase/chromatin remodeling function of SWI2/SNF2-like proteins. This suggests that alterations of chromatin effect global DNA glycosylation patterns in T. brucei. Similar to the involvement of SWI2/SNF2-like proteins in the regulation of DNA methylation, we propose that JBP2 recognizes and binds specific regions of the trypanosome chromosome, hydrolyses ATP, and allows the J-synthesis machinery (i.e., thymine-hydroxylase and glycosyltransferase) to gain access to condensed chromatin by altering chromatin architecture (Figure 7). JBP1 then binds to the basal level of localized J and stimulates additional synthesis by recruitment of the machinery to DNA (Cross et al., 2002). Since the glycosyltransferase is able to convert HOMedU to base J irrespective of the location in the chromosome, it is possible that the stimulatory effect of JBP1 on J biosynthesis is due to the specific recruitment of the thymine-hydroxylase to J DNA. This model of JBP2 function would extend the conservation of chromatin remodeling as a mechanism for regulating DNA modification in mammals and plants to include the regulation of the hypermodified base J in the early branching eukaryote, T. brucei.
JBP2 was identified from the *T. brucei* database based on its homology with the N-terminal third of JBP1 including the putative Myb-like DNA binding domain. Mutagenesis analysis of JBP1 has indicated that residues within the Myb-like domain, as well as regions in the LZ-like motif that are not shared with JBP2, are essential for J DNA binding (C.D. and R.S., unpublished data). Therefore, it’s not surprising that JBP2 may not interact with chromatin in a base J dependent manner. This is consistent with the lack of J DNA binding activity in nuclear extract from the bloodstream JBP1 null (Cross et al., 2002). However, the ability of JBP2 to direct the specific localization of base J synthesis along the chromosome would require the ability of JBP2 to localize along chromatin in a specific manner. Mutations in the conserved Myb-like domain of JBP2, similar to those role of base J in trypanosome DNA, along chromatin in a specific manner. Mutations in the essential nature of JBP2 we will determine the biological significance. Hopefully, by addressing the apparent essential nature of JBP2 we will determine the biological role of base J in trypanosome DNA.

### Experimental Procedures

**Enzymes and Chemicals**

T4 DNA polynucleotide kinase, T4 DNA ligase, and tetracycline were purchased from BRL. Anti-GFP and anti-HA antiserum were from Molecular Probes. AMV reverse transcriptase and Taq polymerase were from Promega. All other chemicals were obtained from Sigma-Aldrich.

**Trypanosome Growth and Transfection**

Bloodstream form trypanosome cell line HN221 of strain 427 of *T. brucei* (Rudenko et al., 1995) were cultured as described (Hirumi et al., 1980). Bloodstream transfections were performed as described previously (Carruthers et al., 1993). Protrusive *T. brucei* strain 29-13, which contains the genes for T7 RNA polymerase and the tetracycline repressor, were cultured in the presence of G418 (15 μg/ml) and hygromycin (50 μg/ml) as described (Wirtz et al., 1999). Wild-type procyclic trypanosomes TREU667 were cultured as previously described (Pollard et al., 1994). Transfection of procyclic cells was performed by resuspending cells (2 × 10^7) in 0.5 ml cytostim (van den Hoff et al., 1992) containing 5 μg of plasmid. Transfections were carried out in 0.4 cm cuvettes using a BioRad electroporator with peak discharge at 1.4 kv and capacitance of 25 μF. Cells were pulsed twice, 10 s apart, and transferred into 10 ml of prewarmed (27°C) media. After 1 day, selection was applied and the cells were grown for 2 wk to form stable lines. Cells were cloned by limiting dilution.

**Expression of JBP1 and JBP2 in *T. brucei***

To allow ectopic expression of JBP1-GFP fusion we utilized a modified pTub-phleo construct (Rudenko et al., 1994) that contains the EGFP ORF cloned between the tubulin flanking sequences. Insertion of the JBP1 gene now allows expression of JBP1-GFP N terminus fusion protein after integration into the tubulin array. JBP1-GFP-tub-phleo was digested with NotI and XhoI before electroporation with bloodstream or procyclic trypanosomes, and transformants were selected for resistance to 2 μg phleomycin per ml. JBP2-GFP-tub-phleo construct and transformants were made as described for JBP1.

To place the JBP1 gene under tetracycline control, a MuLin PCR product from the Tb JBP1 genomic clone was blunt-end cloned into the pHD615 PAC vector digested with BamHI and HindIII and ends filled in with Klenow. pHD615 PAC is based on the plasmid pH615 (Biebing et al., 1997) but contains a puromycin-acetyltransferase-resistance gene instead of a hygromycin-resistance gene (Ulbert et al., 1991). To place an HA tag on the C terminus, the 3′ PCR primer contained an HA-epitope-tag sequence. The final construct, (JBP1-HA-pH615), was linearized with NotI and transfected with *T. brucei* procyclic 29-13 cell line and selected using puromycin at 0.1 μg/ml. To induce JBP1 expression, tetracycline was added at 1 μg/ml.

To produce procyclic cells expressing both JBP1 and JBP2, two sequential transfections were done with the 29-13 cell line using the JBP2-GFP-tub-phleo and JBP1-HA-pH615 constructs followed by selection as described above.
Microscopy
For GFP analysis, cells expressing GFP-fusion proteins were collected by low-speed centrifugation, washed in phosphate buffered saline (PBS) with 5% glucose, DNA stained with DAPI, and allowed to settle on slides coated with 0.8%-1.0% agarose. GFP fluorescence was visualized from live cells illuminated with UV light and a FITC filter using a Zeiss Axiostep 2 microscope equipped with a cooled charged coupled device (CCD) camera.

Western Blotting
Proteins from 10^6 cell equivalents were separated by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDSPAGE; 8% gel), transferred onto nitrocellulose, and probed with the indicated antisera. Bound antibodies were detected by the appropriate secondary antibody conjugated to horseradish peroxidase and visualized by enhanced chemiluminescence (ECL).

RT-PCR
RT reactions were performed on 1 x 10^6 cell equivalents total mRNA from wt TREP 667 procyclic and HN221 bloodstream form cells with AMV reverse transcriptase with 12.5 pmol oligo dT primer for 1 hr at 42°C according to the manufacturer’s instructions. The whole mixture was column purified (QIAGEN, QiAquick) to remove the residual unused oligo dT. Subsequently 2 x 10^5 cell equivalents of each cDNA were used in a PCR amplification using 10 pmol of a conserved 5' spliced leader oligo (Rudenko et al., 1996) (5'-CGCGAGA TTTGCAATTAGATGACTTTTCTA-3') in combination with 10 pmol of a 3' calmodulin oligo (Tsukiyama et al., 1985) (5'-CTCATTCAATTTTGAAGAAC-3'), a 3' JBP1 oligo (5'-GGTTTCTCGTGGCCGAC GAC-3'), or a 3' JBP2 oligo (5'-GCACATGGTAATCATACAC CGCGCTCGTATTC-3') with 2 U Taq polymerase in a 25 μl reaction volume according to the manufacturer’s instructions. Each PCR cycle was 1 min at 94°C, 1 min at 56°C, and 3 min at 72°C followed by 5 min at 94°C and 10 min at 72°C. Samples were taken after the number of PCR cycles indicated. A - RT control was taken for the highest number of cycles used. One-third volume of the PCR was run out on a 1% agarose gel, transferred to a hybridization transfer membrane (Genescreen plus), and hybridized with a full-length calmodulin, JBP1, or JBP2 probe overnight at 42°C in 40% formaldehyde-based hybridization mix containing 10% dextran sulfate. Signals were quantified using a Phosphorimager (Amersham Biosciences).

In this analysis we used two different strains of T. brucei. To address the possibility of point mutation differences between the two strains we would like to acknowledge that: (1) we have confirmed these results using different primers representing different regions of the JBP1 and JBP2 mRNA sequence, and (2) the gene sequences for JBP1 and JBP2 are highly conserved at the DNA-sequence level among different strains of T. brucei. For example, the cloned sequence from the T. brucei 667 is identical to the sequences obtained from the 927 strain. Furthermore, we have confirmed the development-management and low mRNA levels indicated by the PCR analysis of JBP1 by Northern blot analysis (data not shown).

Chromatin Binding
To assay for chromatin interaction, cells expressing either JBP1 of JBP2 were resuspended in lysis buffer (10 mM Tris-HCl [pH 7.9], 250 mM NaCl, 1 mM EDTA, 5 mM CaCl, 10 mM sucrose, 1% Triton X-100, 10% glycerol, and Roche complete proteinase inhibitors according to the manufacturer’s instructions) at 10^6 cell equivalents per μl. After incubation on ice for 15 min, cells were spun at 1000 x g for 5 min in a microcentrifuge, supernatant transferred to a fresh tube, and the pellet resuspended in an equal volume of lysis buffer plus 1% SDS. 10 μl of each fraction was run on an 8% SDS-PAGE and analyzed by Western blot using the indicated antisera.

Determination of the Genomic Level of J
To quantify the genomic level of J, we used the anti-J DNA immuno-noblot assay (van Leeuwen et al., 1997). Briefly, serially diluted genomic DNA was blotted to nitrocellulose and incubated with anti-J antisera. Bound antibodies were detected by a secondary antibody conjugated to horseradish peroxidase and visualized by ECL. The membrane was stripped and hybridized with a probe for the tubulin gene array to correct for DNA loading. This assay allows us to detect less that one J in 10^6 bases (van Leeuwen et al., 1997).

Determination of the Sequence Distribution of J
This was done as previously described (van Leeuwen et al., 1997, 2000; Cross et al., 2002).

Acknowledgments
We are grateful to Torsten Ochsenreiter, Justin Widarter, Elisabetta Ullu, Piet Borst, and Steve Hajduk for critically reading the manuscript. We would like to thank Kevin Morgan for assistance with early experiments on this project. We would also like to thank Piet Borst, Steve Hajduk, Jayleen Grams, and members of the Borst and Hajduk lab for their helpful advice and support. We thank Christiane Tschudi and Paul Andre Gene for providing the JBP1-β-A-hPD615 and JBP1-GBP-tub-pheo clones, respectively. Anti-J antisera was a gift from Piet Borst. We are grateful to Elisabetta Ullu and Christian Tschudi for providing anti-La. The 70 bp probe was a gift from Richard McCulloch.

Received: September 29, 2004
Revised: November 16, 2004
Accepted: December 10, 2004
Published: February 3, 2005

References


