Epigenetic Regulation of Polymerase II Transcription Initiation in Trypanosoma cruzi: Modulation of Nucleosome Abundance, Histone Modification, and Polymerase Occupancy by O-Linked Thymine DNA Glucosylation

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Trypanosoma cruzi is a major human parasitic pathogen with a complex life cycle, alternating between an insect vector and mammalian hosts (42). In both, T. cruzi undergoes differential morphological and functional changes that require rapid and selective changes in gene expression profile. Unlike other eukaryotes, the genes in T. cruzi are arranged in large polycistronic transcription units (PTUs). On the basis of this gene organization, it was previously thought that trypanosomes rely solely on posttranscriptional processes to regulate gene expression. We recently localized a novel glucosylated thymine DNA base, called base J, to potential promoter regions of PTUs throughout the trypanosome genome. Loss of base J, following the deletion of JBP1, a thymidine hydroxylase involved with synthesis, led to a global increase in the Pol II transcription rate and gene expression. In order to determine the mechanism by which base J regulates transcription, we have characterized changes in chromatin structure and Pol II recruitment to promoter regions following the loss of base J. The loss of base J coincides with a decrease in nucleosome abundance, increased histone H3/H4 acetylation, and increased Pol II occupancy at promoter regions, including the well-characterized spliced leader RNA gene promoter. These studies present the first direct evidence for epigenetic regulation of Pol II transcription initiation via DNA modification and chromatin structure in kinetoplastids as well as provide a mechanism for regulation of trypanosome gene expression via the novel hypermodified base J.

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While these studies provided the first direct evidence for epigenetic regulation of gene expression in kinetoplastids at the level of Pol II transcription, they do not explain how base J regulates transcription.

Transcription in eukaryotes is influenced by chromatin structure. In particular, the packaging of DNA into chromatin imposes significant obstacles to transcription initiation by Pol II (18, 24). The structure of chromatin can regulate the binding of proteins/complexes to promoter regions, including the recruitment of Pol II and formation of the preinitiation complex. Thus, chromatin is highly dynamic and continuously shifting between an open transcriptionally active conformation and a compact silenced one (18). This fluid nature of chromatin is tightly regulated through multiple mechanisms, including histone modification, nucleosome density, and DNA methylation (24). Global mapping of nucleosomes in Saccharomyces cerevisiae yeast demonstrates that upon gene activation nucleosomes are evicted at many promoters (1, 23, 33) and are reassembled upon gene silencing (14, 38). Histone acetylation is associated with an increase in the accessibility of DNA to transcriptional machinery (13). Acetylation of lysine residues at the N-terminal domain of histones H3 and H4 weakens the interactions with DNA and results in a destabilization of nucleosomal structures and activation of gene transcription (27, 43). Accordingly, increased histone acetylation at the promoter region has been linked to activation of transcription, where the level of acetylation is proportional to the transcription rate (24, 31).

Genome-wide chromatin immunoprecipitation (ChIP) studies of Trypanosoma brucei and Leishmania major found two peaks of acetylated H4K10 enrichment at every divergent SSR, marking initiation (39, 41). Similarly, in T. cruzi, acetylated H3K9/H3K14, H4K10, and methylated H3K4 are enriched at divergent SSRs, with a bimodal profile demarcating the bidirectional transcription initiation sites (34). Therefore, the presence of these multiple posttranscriptional modifications of histones has been implicated in the regulation of transcription initiation in kinetoplastids.

Modulation of transcription initiation sites of many organisms is regulated by cooperative interaction of epigenetic modifications of DNA and histone (22, 32). The presence of 5-methylcytosine within chromatin ensures robust transcriptional silencing, as it can directly inhibit transcription factor binding (47) and act as a binding platform for recruiting histone-modifying and chromatin-remodeling proteins, including the histone deacetylase corepressor complex (29, 46). Regulation of nucleosome density, histone modification, and DNA modification are therefore important mechanisms to enable the transcription machinery to access the DNA.

Similar to the mechanism of epigenetic modifications in other eukaryotes, we predicted that the presence of base J at promoter regions alters chromatin structure and changes the accessibility of DNA binding proteins, including RNA polymerase II, thereby regulating transcription initiation and trypanosome gene expression. Therefore, we analyzed the structure and abundance of modified and unmodified histones of T. cruzi chromatin following the loss of base J. We find that the loss of base J coincides with decreased nucleosome abundance, increased histone H3/H4 acetylation, and increased Pol II occupancy at transcription initiation sites. These studies present the first experimental evidence to directly support the role of epigenetics and chromatin structure in the regulation of Pol II transcription in kinetoplastids. Furthermore, we now provide a mechanism for the novel hypermodified base J regulation of Pol II transcription initiation in T. cruzi.

MATERIALS AND METHODS

Growth of cells. Y strain T. cruzi wild-type (WT) and JBPI double-KO (JBPI dKO) epimastigotes were grown in liver infusion tryptose (LIT) medium containing 10% fetal bovine serum as previously described (4). Epimastigotes from both WT and KO strains were harvested at mid-log phase by centrifugation for subsequent analysis.

FAIRE analysis. Formaldehyde-assisted regulatory element (FAIRE) analysis was performed as previously described (16). Briefly, 1 × 10^7 T. cruzi cells were fixed in 1% formaldehyde for 5 min in LIT medium, and the reaction was terminated by adding 2.5 M glycine to a final concentration of 125 mM. Fixed cells were then lysed by adding cell lysis solution containing 10 mM EDTA, 50 mM Tris-HCl, 1% SDS, and protease inhibitors. DNA was sonicated with a sonic amplitude sonicator for 10 min (30 s on/off cycles) in order to obtain chromatin fragments of an average of 500 bp in length. Debris was removed by centrifugation. A non-cross-linked sample was obtained for each replicate as a total DNA control. DNA from both cross-linked and non-cross-linked samples was extracted by two consecutive phenol-chloroform extractions, and DNA was ethanol precipitated in the presence of 20 μg/ml of glycogen after RNA and proteinase K treatment. Each experiment was performed in triplicate, and quantification of the FAIRE and total DNA samples was performed by real-time PCR, as described below, and normalized to the analysis of 24S rRNA gene (a non-Pol II transcribed locus that lacks base J). The primer sequences utilized in all analyses will be provided upon request.

ChIP analysis for histone and Pol II. ChIP was performed as previously described (16). Briefly, DNA was cross-linked to protein using formaldehyde as in FAIRE analysis. Sonicated DNA extract was precleared using protein A agarose beads and incubated with or without relevant antibodies. Chromatin from 7 × 10^7 cell equivalents was used in each immunoprecipitation (IP) reaction. Lysate was incubated with commercially prepared anti-histone H3 (AbCam) at a concentration of 2 μg per immunoprecipitation reaction, anti-acetylated H4 (anti-AcH4; AbCam) at a concentration of 5 μg per IP reaction, or anti-acetylated H3 (anti-AcH3) at a concentration of 5 μg per IP reaction overnight at 4°C. T. cruzi RNA Pol II C-terminal domain antibodies (a kind gift from Sergio Shenkman) were used at 10 μg per IP reaction. Protein-DNA complexes were incubated with protein agarse A beads for 2 h and washed 3 times using wash buffers containing 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, 500 to 150 mM NaCl, and protease inhibitors. DNA was eluted from beads using elution buffer containing 0.1% SDS and 0.1 M NaHCO_3. Cross-linking was then reversed by adding NaCl to a final concentration of 325 mM, and DNA was incubated at 65°C overnight. DNA was then extracted using phenol-chloroform after RNase and proteinase K treatments. Each ChIP experiment was performed in triplicate and quantified by quantitative real-time PCR (RT-PCR).

Quantitative RT-PCR. Input and immunoprecipitated DNA was obtained from three biological replicates from WT and KO cells as described above. Quantification was performed on an iCycler instrument with an iQ5 multicolor real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). The primer sequences used in the analysis are available upon request. Reaction mixtures contained 5 pmol forward and reverse primers, 2× iQ SYBR green Super Mix (Bio-Rad Laboratories, Hercules, CA), and 2 μl of template cDNA. Standard curves were prepared for each gene using 10-fold dilutions of a known quantity (15 ng/μl) of WT DNA. Quantifications were calculated using iQ5 optical detection system software. The value for each sample was normalized to the 24S rRNA value. The average ratios were calculated for each gene using the average of three biological replicates for each analysis.

RESULTS

Base J regulates nucleosome abundance and histone acetylation at divergent Pol II transcription initiation sites. We and others have demonstrated that divergent SSRs in T. cruzi contain bidirectional promoters (11, 34). Our previous studies demonstrated the presence of base J at the divergent and convergent SSRs, and the loss of base J from these regions in
JBP1 dKO cells leads to increased Pol II transcription with no defects in termination (11). We concluded that base J specifically regulates chromatin structure at Pol II transcription initiation sites. To investigate this possibility, we first looked at the nucleosome occupancy in SSRs using FAIRE analysis of WT and JBP1 dKO cells. FAIRE analysis identifies nucleosome-depleted or naked regions such as highly transcribed promoter regions and has previously been utilized to characterize the chromatin landscape of Pol I, II, and III loci in trypanosomes (16, 28). When formaldehyde-cross-linked and sheared chromatin is extracted with phenol-chloroform, fragments of DNA that were naked or contained loosely associated histones or other proteins are enriched in the aqueous phase.

We initially scanned a SSR by quantitative PCR (qPCR) to amplify 200-bp fragments covering the divergent SSR as well as regions extending into the adjacent polycistronic unit (Fig. 1). Our previous studies mapped the approximate location of the divergent transcription initiation sites in this SSR and demonstrated that the loss of base J leads to an increased rate of Pol II transcription of the adjacent PTUs (10). We now demonstrate increased FAIRE enrichment in the JBP1 dKO cells, corresponding to increased soluble chromatin, as we progress into the SSRs, with a peak approximately in the center and adjacent to the mapped transcription initiation sites. We then analyzed the center of three additional divergent SSRs within the T. cruzi genome (Fig. 2A). We have previously demonstrated the loss of base J in these SSRs in the JBP1 dKO cell line, and further analysis of one (SSR 7617) confirmed that this led to an increased rate of PTU transcription (11). Overall, we found that divergent SSRs were significantly more enriched in the aqueous phase in the transcriptionally activated JBP1 dKO cell line than in the WT cell line, consistent with the depletion of nucleosomes at transcription initiation sites following the loss of base J (Fig. 2A; see Fig. S1 in the supplemental material). In contrast, there was no change at convergent SSRs between JBP1 dKO and WT cell lines (Fig. 2B). The low level of soluble chromatin at convergent SSRs compared with divergent transcription initiation sites (20- to 60-fold less enrichment of convergent regions in WT cells) indicates increased levels of protein bound to DNA involved in transcriptional termination than regions involved in initiation.

We next performed ChIP using formaldehyde-cross-linked chromatin and anti-histone H3 antibodies followed by qPCR to directly characterize the nucleosome density within various SSRs. As reported previously in trypanosomes, histone H3 content is a good indicator of nucleosome density (16, 26, 40). Consistent with the results of FAIRE analysis, we see a significant reduction in H3 occupancy at divergent SSRs of JBP1 dKO cells compared to WT cells and no change at convergent SSRs (Fig. 2C and D). Overall these data suggested a significant change in chromatin structure of Pol II transcription initiation sites upon the loss of base J. However, the loss of base J has no discernible effect on the protein-rich chromatin representing transcription termination sites.

Histone acetylation is almost invariably associated with activation of transcription. Previous studies showed an enrichment of acetylated histone H3 and H4 at Pol II transcriptional start sites in kinetoplastids (34, 39). Furthermore, acetylated histones H3 and H4 are components of active promoters in the protozoan parasite Toxoplasma gondii (17, 37). In order to investigate if base J affects the acetylation of core histones at transcription initiation sites, we performed a ChIP assay using anti-AcH3 and anti-AcH4 antibodies in WT and JBP1 dKO cells. Although nucleosome abundance is reduced at divergent SSRs, the fraction of acetylated H3 and H4 histones increased significantly in the JBP1 dKO cell line (Fig. 2E and G). Convergent termination regions contain lower levels of acetylated histones than divergent transcription initiation sites, with no
change in levels upon the loss of base J (Fig. 2F and H). Lower levels of acetylated H3 and H4 in termination regions than divergent SSRs in *T. cruzi* have been characterized previously (34).

From the results of FAIRE analysis and the histone ChIP experiments described above, we conclude that the loss of base J from transcription initiation sites of Pol II transcribed gene clusters leads to decreased nucleosome abundance and increased H3 and H4 acetylation of the remaining nucleosomes. Furthermore, acetylated nucleosomes were preferentially localized at divergent transcription initiation sites rather than termination sites. These results are consistent with our earlier conclusion that the modulation of chromatin structure by base J regulates Pol II transcription initiation and genome-wide changes in gene expression (11). The finding that loss of base J does not lead to any detectable changes in chromatin structure at convergent termination sites is also consistent with our inability to detect defects in Pol II transcription termination in the JBP1 dKO cell line (11).

**Chromatin changes at the SL RNA promoter/locus upon loss of base J.** In trypanosomes, maturation of polycistronic mRNAs requires the addition of a 39-nucleotide spliced leader (SL) RNA onto the 5' end of every mRNA (25). In order to provide the large amount of SL RNA required for trans-splicing, the trypanosome has up to 200 SL RNA transcription units that are transcribed by Pol II. The SL RNA gene contains the only defined Pol II-dependent promoter in trypanosomes (30). In fact, all knowledge of proteins and DNA sequences essential for Pol II-dependent transcription in kinetoplastids comes from SL RNA gene studies. Therefore, to fully understand the role of base J in regulating Pol II transcription, we analyzed the SL RNA gene array. Figure 3A depicts an SL RNA gene and promoter array, a region extending ~1 kb downstream of the SL RNA gene array.
stream (and upstream) of the promoter, and CZAR (*cruzi*-associated retrotransposon) represents a nontranscribed retrotransposable element which has integrated exclusively within the SL RNA array. Using anti-base J IP, we have localized base J within the SL gene array of WT (*T. cruzi*) (Fig. 3 B). As expected, base J was absent from these regions in the JBP1 dKO cell line. This loss of base J leads to a 2.33-fold increase in the rate of Pol II transcription of the SL RNA gene (6). In order to characterize changes in the chromatin environment of a defined Pol II promoter resulting from the loss of base J and explain the increased SL transcription, we performed FAIRE and ChIP analyses of the SL array as described above. We found an increase in abundance of the soluble fraction of chromatin at the SL promoter and nontranscribed spacer region of JBP1 dKO cells compared to WT cells. Consistent with inactive regions of chromatin, there was approximately 10-fold less soluble chromatin present at the CZAR repeats of WT cells than the SL promoter and no significant change in the JBP1 dKO cells (Fig. 3C). Consistent with FAIRE analysis, we found approximately 2-fold less histone H3 present at the SL promoter and nontranscribed spacer regions (Fig. 3D). The initial low abundance of histone H3 at the SL promoter/RNA gene in WT cells is consistent with the findings of previous studies in *Leishmania*, *T. brucei*, and *T. cruzi* (19, 34, 40). We also detected a significant increase in acetylated histone H3 and H4 abundance at the SL gene and nontranscribed spacer region in the JBP1 dKO cell line (Fig. 3E and F). More significant changes in H3 and H4 acetylation are evident at the SL gene/promoter region than ~1 kb away in the nts. Taken together, we can conclude that loss of base J leads to changes in chromatin structure and increased H3 and H4 acetylation at the defined SL Pol II promoter as well as undefined promoters within SSRs between divergent gene clusters throughout the *T. cruzi* genome.

**Increased RNA polymerase II occupancy following loss of base J.** Eukaryotic gene expression begins with recruitment of the transcription machinery to a gene promoter and formation of a preinitiation complex composed of Pol II and general transcription factors (35). To evaluate the impact of base J on the formation of the preinitiation complex *in vivo*, we investigated the changes in Pol II promoter occupancy upon depletion of JBP1 by ChIP using specific antibodies to
the \textit{T. cruzi} Pol II unique carboxy-terminal domain (8). Initially, we analyzed Pol II occupancy within a divergent SSR using ChIP/qPCR as previously performed with FAIRE analysis (Fig. 4A). We found that the highest levels of Pol II within the SSR flank the previously mapped bidirectional transcription initiation sites, similar to the nucleosome depletion profile (Fig. 1). Loss of base J in the JBP1 dKO cells led to a 2- to 4-fold increase in Pol II occupancy at these transcription initiation sites within the divergent SSR (Fig. 4A), as well as a 3- to 6-fold increase within three additional SSRs (Fig. 4B).

We then extended our analysis to determine the Pol II accumulation at the defined Pol II-dependent SL promoter in \textit{T. cruzi}. As expected, in both WT and JBP1 dKO cell lines we saw higher levels of Pol II at the promoter than the SL gene and an almost complete absence of the polymerase at the nontranscribed spacer and CZAR retrotransposon. However, we detected an ~3-fold increased accumulation of Pol II at the SL promoter and gene in the JBP1 dKO cells (Fig. 4C). Overall, the changes in Pol II occupancy correlated with the increased rate of nascent mRNA formation within the adjacent polycistronic units and SL RNA gene following the loss of base J (11). These results suggest that base J regulates chromatin structure and the formation of the Pol II preinitiation complex.

**DISCUSSION**

In eukaryotes, transcription initiation is a key regulatory point in controlling the level of gene expression. Transcription initiation involves a series of events, including protein modification (i.e., histone), chromatin remodeling, binding of specific proteins to DNA (i.e., activators), and recruitment of Pol II. However, previous studies of trypanosome gene expression indicated that genes are not activated at the transcriptional level (5). The arrangement of functionally unrelated genes in polycistronic units and the absence of classic Pol II promoters led to the belief that transcription initiation was not a key factor in regulating trypanosome mRNA production. Localization of specific modified and variant histones at SSRs in \textit{T. cruzi} development (12) suggest a role for epigenetics in control of gene expression. Chromatin has recently been shown to be involved in the regulation of the RNA Pol I transcribed protein-coding genes within the telomeric gene cluster in \textit{T. brucei}, including the variant surface glycoprotein (VSG) genes. Activation of the Pol I gene cluster correlates with nucleosome depletion (16, 40), and proteins involved in chromatin remodeling or histone modification play a role in silencing (15, 20). However, no direct evidence for epigenetic regulation of Pol II transcribed genes has been provided. We recently described a novel epigenetic modifica-
tion of DNA that consists of the hydroxylation and glucosylation of the thymine base of DNA, called base J, at Pol II transcription initiation sites and that, upon removal, leads to an increased rate of mRNA transcription. Accordingly, we now demonstrate that the loss of base J at Pol II transcription initiation sites leads to distinct changes in chromatin structure/modification and increased occupancy of Pol II. These studies provide the first evidence that Pol II transcription initiation and key regulatory mechanisms, including histone modification, chromatin remodeling, and recruitment of polymerase, are regulated in an epigenetic fashion in trypanosomes.

Chromatin structure needs to be dynamically modulated in order to allow transcription and regulate cellular function. This is especially true for single-celled organisms that need to rapidly adapt to changing environmental growth conditions (i.e., the host environment) during their life cycle and is reflected in changes in gene expression. Chromatin structure has been shown to regulate all stages of transcription initiation in eukaryotes, including the binding of activators upstream of the core promoter, recruitment of complexes that facilitate the binding of general transcription factors, recruitment of Pol II, and formation of the preinitiation complex. However, very little is understood regarding the regulation of Pol II initiation, including the role of chromatin, in trypanosomes. Here, we identify that even in these early-divergent eukaryotes, DNA modification, histone modification, and nucleosome remodeling/abundance work together to regulate chromatin structure and Pol II transcription.

According to the histone code hypothesis, modification of histone serves as a recognition platform for binding of factors such as chromatin-remodeling proteins (24). It has been demonstrated in yeast that covalent modification of histones may precede their dissociation from DNA (33, 49). Furthermore, histone acetylation serves as a mark for actively transcribed chromatin, where changes in the net charge of the histone due to the acetyl group presumably lead to unfolding of the nucleosomal fiber, rendering regulatory sites more accessible (9). We demonstrate here that the loss of base J is followed by the reduction of nucleosomes and increased acetylation of histones H3 and H4 at Pol II promoter regions. Since the C-terminal region of H2A is also acetylated in trypanosomes (21) and the antisera that we utilized show some cross-reactivity (34), we cannot rule out the involvement of acetylated histone H2A in this model. Regulation of nucleosome density and histone modification are therefore highly conserved mechanisms to enable the transcription machinery to access the DNA. Furthermore, it follows that in the case of trypanosomes, the acetylation of promoter-associated nucleosomes occurs prior to their disassembly. We propose that the loss of base J leads to increased accessibility of DNA binding proteins, including histone-modifying factors. The modification of histone tails, including acetylation of H4/H3 at Pol II promoters, eventually leads to a decreased abundance of nucleosomes and increased recruitment of Pol II, resulting in an increased rate of transcription initiation. In contrast, the loss of base J at tightly closed chromatin, such as regions involved in Pol II transcription termination, fails to affect the recruitment of histone-modifying factors. FAIRE analysis indicates increased localization/binding of proteins within the convergent SSRs. Whether this is due to a specific increase in nonnucleosomal proteins needs to be determined. Therefore, the removal of base J in these regions had no measurable effect on chromatin structure or the ability to properly terminate Pol II transcription (11). It follows that in certain contexts, such as relatively relaxed chromatin structures at promoter regions, base J is a key factor regulating the formation of repressive chromatin. On the basis of the data presented here, the level of base J at these sites directly correlates with the degree of repressed chromatin and reduced preinitiation complex formation. However, at regions where closed chromatin is essential, such as regions involved in transcription termination, base J is not a significant factor. Future studies will address the mechanism of base J regulation of Pol II initiation in more detail.

While we clearly demonstrate epigenetic mechanisms that regulate Pol II occupancy and transcription initiation in trypanosomes, it is difficult to propose a model in which transcription of the PTUs is tightly regulated rather than constitutive. Unlike prokaryotic operons, there is no clear functional relationship among genes within a PTU in trypanosomes. Therefore, there is no obvious benefit to the parasite to differentially regulate transcription of individual PTUs. However, we have previously shown that the loss of base J at Pol II transcription initiation sites and increased transcription of PTUs lead to significant up- and downregulation of gene expression in T. cruzi (10). Apparently, the regulation of transcription via base J works in conjunction with established posttranscriptional regulatory mechanisms. Increased transcription throughout the PTU still results in reduced steady-state concentrations of certain mRNAs. We have demonstrated in T. cruzi that the loss of JBP2 function did not lead to the loss of base J from every PTU Pol II transcription initiation site and that the loss of each JBP enzyme led to differential changes in gene expression (10). Therefore, a combination of differential regulation of JBP function and levels of base J at PTU and posttranscriptional regulatory mechanisms may provide a mechanism to fine-tune trypanosome gene expression and to potentiate genes for further or future activation and/or inactivation. Furthermore, the analysis of JBP thymine hydroxylase activity has demonstrated the sensitivity of base J synthesis to levels of iron, 2-oxoglutarate, succinate, and oxygen, providing a link between parasite metabolism, host environment, and regulation of gene expression and pathogenesis (L. Cliffe, D. Ekanayake, G. Hirsch, M. Hu, and R. Sabatini, unpublished data).

In summary, we have provided the first direct evidence for epigenetic regulation of Pol II transcription initiation via DNA modification and chromatin structure in early-branaching trypanosomes. Future studies are required to identify how the unique hypermodified DNA base J regulates chromatin structure and nucleosome modifications and why these organisms need to regulate transcription initiation at large polygene clusters throughout the genome.

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