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Base J Glucosyltransferase does not regulate the sequence specificity of J synthesis in trypanosomatid telomeric DNA

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Abstract

Telomeric DNA of Trypanosomatids possesses a modified thymine base, called base J, that is synthesized in a two-step process; the base is hydroxylated by a thymidine hydroxylase forming hydroxymethyluracil (hmU) and a glucose moiety is then attached by the J-associated glucosyltransferase (JGT). To examine the importance of JGT in modifiying specific thymine in DNA, we used a *Leishmania* episome system to demonstrate that the telomeric repeat (GGGTTA) stimulates J synthesis *in vivo* while mutant telomeric sequences (GGGTTT, GGGATT, and GGGAAA) do not. Utilizing an *in vitro* GT assay we find that JGT can glycosylate hmU within any sequence with no significant change in Km or kcat, even mutant telomeric sequences that are unable to be J-modified *in vivo*. The data suggests that JGT possesses no DNA sequence specificity *in vitro*, lending support to the hypothesis that the specificity of base J synthesis is not at the level of the JGT reaction.

Graphical abstract

in vivo only particular DNA sequences can become J modified. The specificity of J modification is not due to the JGT.

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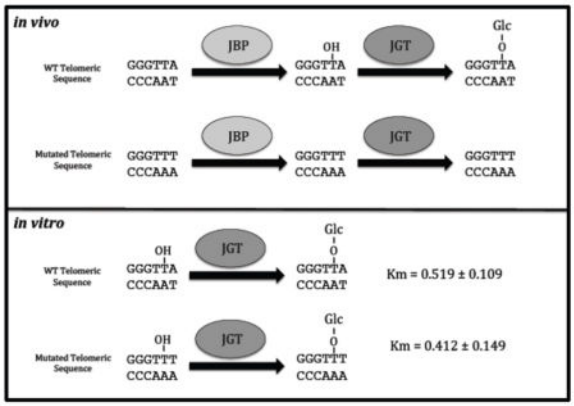

Keywords

Glucosyltransferase; base J; Trypanosomatid; epigenetic; RNA Polymerase II transcription

Trypanosomatids, including the human pathogens *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Leishmania*, possess a unique DNA modification within their genomes known as base J [1, 2]. Base J (β-D-glucopyranosyloxymethyluracil) is a hyper-modified thymine residue predominately present in repetitive sequences, such as telomeric repeats [2, 3]. While the function of base J in telomeric repeats is unknown, base J is also found at chromosome-internal regions at regions flanking polycistronic transcription units called divergent strand switch regions (dSSRs) and convergent strand switch regions (cSSRs), which are sites of RNA polymerase II (Pol II) transcription initiation and termination, respectively [4]. The loss of base J from these chromosome-internal sites led to alterations in transcription initiation and termination, and corresponding changes in gene expression [5–7]. While it is clear that base J represents a novel epigenetic mark involved in regulating Pol II transcription and gene expression, little is understood about what regulates the specific localization of base J in the genome.

Base J is synthesized in a two-step process. First, thymine residues in the context of DNA are hydroxylated by a thymidine hydroxylase (TH) forming 5-hydroxymethyluracil (hmU). A glucose moiety is then attached to hmU by a glucosyltransferase (JGT), to form base J. Two TH enzymes, JBP1 and JBP2, have been identified in trypanosomatids [8–11]. While both JBP1 and JBP2 stimulate *de novo* thymidine hydroxylation *in vivo*, the ability of JBP1 to bind J-DNA is thought to play a unique role in J propagation/maintenance [10, 12–14]. The simultaneous deletion of both JBP1 and JBP2 from *T. brucei* yields a cell line that is unable to synthesize base J, unless cells are fed hmU [9]. Studies such as these unambiguously identified JBP1/2 as the thymidine hydroxylases catalyzing the first step of base J synthesis as well as confirm that this step is independent from the subsequent step of glucose conjugation during base J synthesis. The glucosyltransferase involved in the second step of base J synthesis, base J associated GT (JGT), has recently been identified [15, 16]. Using recombinant protein we demonstrated that JGT utilizes UDP-glucose to transfer glucose to dsDNA substrates containing hmU [15]. *In vivo*, deletion of both JGT alleles in *T. brucei* results in complete loss of base J synthesis in the genome [15, 16]. These studies

further confirm the two-step mechanism of J synthesis and indicate JGT is the only glucosyltransferase involved, catalyzing the final step of base J synthesis.

Despite the recent genome-wide data sets of DNA Jaylation patterns, and elucidation of the J-biosynthetic pathway, the rules that govern the establishment of DNA J patterns in trypanosomatids remain undefined. It is unclear what determines the specific localization of base J synthesis into specific sequences in the genome. While no consensus sequence or motif is evident from the genome-wide J analysis thus far, it is clear that, for at least the telomeric repeats, there is a sequence specificity component where in the top strand (GGGTTA), only the second T is modified [17, 18].

To gain systematic insight into the constraints that define endogenous Jaylation patterns, we integrated different DNA elements into episomes in *L. major*. To examine the ability of specific sequences to stimulate *de novo* J synthesis, DNA fragments were cloned into a PSP72 vector containing a hygromycin resistance gene and then transfected into *Leishmania major* and grown as episomes (Figure 1a). The episomes were then purified from *L. major*, digested with EcoRI and HindIII, and J synthesis assayed by anti-J IP-qPCR. This set up allows us to control for chromosomal environment and potential indirect effects such as those mediated by transcription. Furthermore, this approach allows us to measure the contributions of DNA sequence in establishing Jaylation patterns. To determine if the episome system would mimic the specificity of J synthesis *in vivo*, sequences representing J positive and J negative regions of the *L. major* genome were cloned into the PSP72 vector and assayed for base J synthesis after growth in *L. major*. Initially, this included J positive and J negative SSRs. Approximately 1kb genomic fragments representing a cSSR that normally contains base J (cSSR+) and a cSSR that lacks base J (cSSR-) were cloned into the PSP72 vector [6]. A similar approach was employed for dSSRs that do and do not contain J *in vivo*. In both cases, only the sequences that contain J *in vivo* are able to stimulate *de novo* J synthesis when cloned in the episome (Figure 1b and c). Thus, even when present outside of their normal chromatin context, i.e. within an episome instead of within the genome, DNA sequence specificity of J synthesis is maintained. This was also seen using a similar approach in *L. tarentolae* [17].

To further address the DNA sequence specificity, we assayed the telomeric repeat sequence in the episome system. A plasmid was generated that contains six copies of the WT telomeric repeat sequence (GGGTTA) and transfected into *L. major*. As expected, WT telomeric repeat sequence (GGGTTA) was able to stimulate J synthesis (Figure 1d). However, mutated telomeric sequences (GGGTTT, GGGATT, or GGGAAA) were unable to support J synthesis (Figure 1d). These data demonstrate that some aspect of J synthesis is DNA sequence specific, at least within telomeric DNA.

Taken together, these findings indicate that DNA Jaylation is largely regulated by cis-acting sequences and is thus genetically encoded. A functional role for interplay between DNA methylation and chromatin has been well established, making it possible that chromatin could be a determining factor in establishing global J patterns in trypanosomes. While these findings do not exclude the possibility that chromatin structure or other associated proteins

are crucial in mediating local DNA Jaylation, they do show that local DNA sequence is a primary determinant of target specification for DNA Jaylation in trypanosomatids.

The current hypothesis is that the key regulatory step of J synthesis is the first step catalyzed by JBP1 and JBP2. Bypassing this first step, via feeding cells hmU, leads to J synthesis in regions of the genome that do not normally contain base J [9, 19]. This suggests that, regardless of where hmU is present, JGT will convert it to base J in a promiscuous (non-sequence-specific) manner. Thus, it follows that the specificity of base J localization is due to the JBP enzymes generating hmU at only specific sites throughout the genome; however, no direct evidence has confirmed this hypothesis. To address this, we tested synthetic telomeric DNA substrates in an *in vitro* GT assay to determine whether JGT can explain the sequence specificity of J synthesis *in vivo*. We took advantage of active recombinant JGT and an *in vitro* homogeneous bioluminescent UDP detection assay (UDP-Glo) that can detect the activity of glycosyltransferases that utilize UDP-sugars and release UDP as a product (see Supplemental Data 1 for details on methods). Using this assay we show that JGT is specific for hmU-containing dsDNA substrates and has no activity with unmodified dsDNA substrates (Figure 2b), consistent with assays directly measuring the transfer of glucose to DNA (Figure 2a), validating our use of UDP-Glo for further *in vitro* GT experiments.

To address sequence specificity, recombinant JGT was incubated with dsDNA substrates that correspond with the WT telomeric sequence (GGGTTA) as well as mutated sequences (GGGTTT and GGGATT) with an hmU positioned on the second T in the G-rich strand (WT_2, TTT, ATT; Table 1). Michaelis-Menten curves were generated for each substrate (Figure 2b and data not shown) and hmU DNA kinetic parameters were determined (Table 1). We found no significant difference in either affinity (Km), p value > 0.05 or turnover (kcat), p value > 0.01 of the JGT when incubated with WT or mutated telomeric sequences. We also find no significant difference in affinity or turnover for WT telomeric sequences with hmU modification at different positions, including the first T on the G-strand that is not modified *in vivo* (WT_1), or for hmU within a random (non-telomeric) DNA sequence (Random; Table 1). These data indicate that JGT is DNA sequence non-specific and thus, the enzyme presumably does not contribute to the telomeric DNA sequence specificity of J synthesis we characterized *in vivo*.

These studies pave the way for the elucidation of the underlying molecular mechanisms involved in regulating J synthesis *in vivo*. While the results described here strongly support the importance of the first step of J synthesis, further work is required to fully understand the formation of hmU on specific sequences by the JBPs. Similar analysis of JBP catalysis, as we performed here for JGT, will help to shed light on primary DNA sequence requirements for hmU formation. *In vitro* analysis of the sequence specificity of the JBPs has, however, proven to be quite difficult. Currently, we are only able to express recombinant protein for JBP1. All attempts to express JBP2, the key *de novo* J synthesis enzyme, have failed. While we have had success demonstrating dioxygenase activity of JBP1 *in vitro*, the reaction is extremely inefficient [20]. Until we have a clear and robust assay for both hydroxylases, we are unable to fully characterize the contribution of this first step in determining the sequence specificity of J synthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Base J	beta-D-glucopyranosyloxymethyluracil
TH	thymidine hydroxylase
JGT	base J-associated glucosyl transferase
JBP	base J-binding protein
UDP-Glc	uridine diphosphoglucose

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Highlights

- Only specific DNA sequences can promote base J synthesis *in vivo.*
- Base J associated glucosyltransferase does not possess DNA sequence specificity.
- Specific localization of base J throughout the genome is likely not due to the glucosyltransferase catalyzing the second step of base J synthesis.

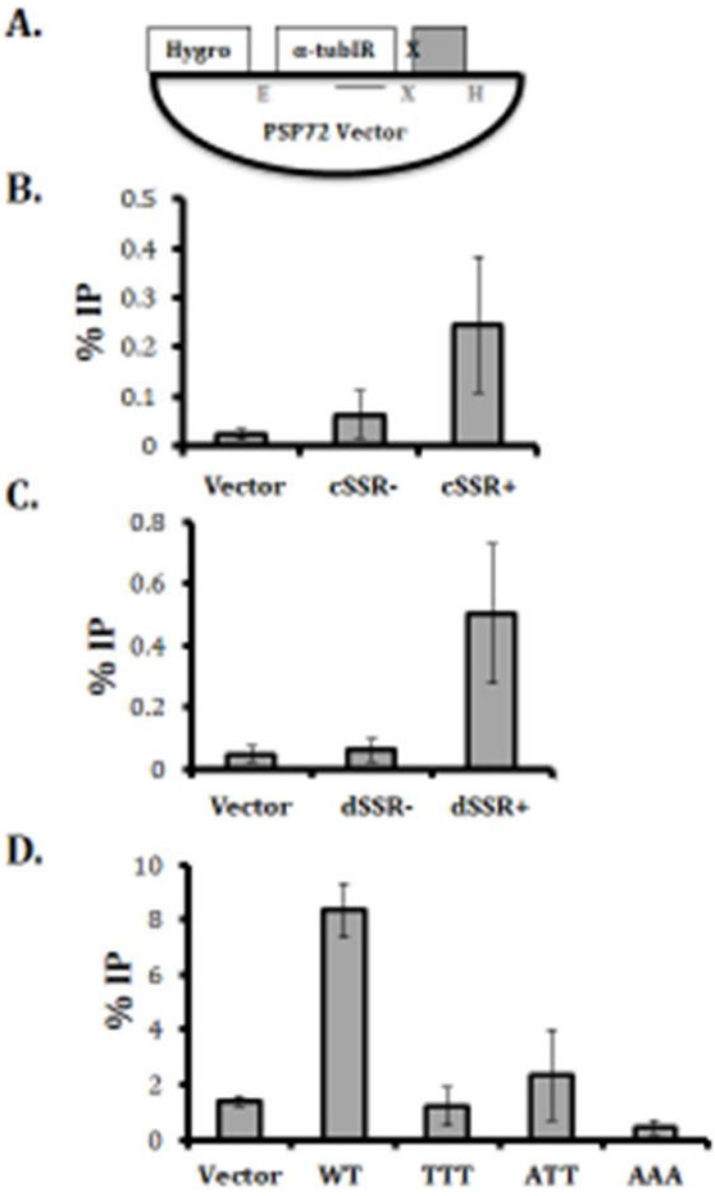

Fig. 1. Specific DNA sequences promote base J synthesis *in vivo*

(A) Schematic of the plasmids containing strand switch regions and telomeric repeats. Fragments, indicated by the grey box, corresponding to ~1kb regions from SSRs and telomeric repeats were cloned into the XhoI (X) and HindIII (H) restriction site of the PSP72 Vector before transfection into wild type *L. major*. The hygromycin resistance gene (Hyg) proves a selectable marker after transfection. Plasmids were digested with EcoRI (E) and HindIII (H) and J levels stimulated by the cloned DNA fragment was determined by anti-J IP-qPCR as described in supplementary materials and methods. Solid line below tub IR indicates region amplified in qPCR. (B–D) The percent IP of J-containing DNA from an empty PSP72 vector, and PSP72 vector containing a SSR fragment and telomeric repeats. %IP was calculated relative to input DNA. (B) Convergent SSR that lacks base J (cSSR−) or contains base J (cSSR+) in the normal genomic context. (C) Divergent SSR that lacks base J

(dSSR−) or contains base J (dSSR+) in the normal genomic context. (D) % J DNA IP resulting from 6 copies of the wild type GGGTTA telomeric sequence; GGGTTT (TTT), GGGATT (ATT), GGGAAA (AAA). Experiments were performed in triplicate and error bars are representative of standard error.

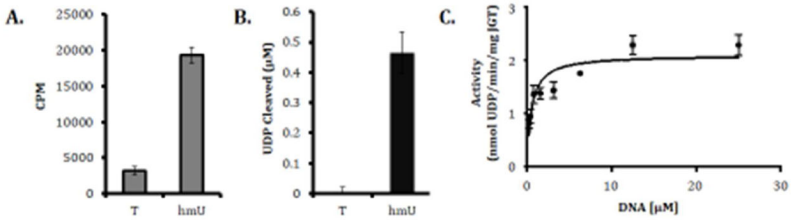

Fig. 2. JGT is specific for hmU containing DNA but does not possess sequence specificity

(A) Radiolabeled *in vitro* glucosyltransferase reaction. Recombinant JGT and UDP-[^{3}H] glucose was incubated with 36nt long dsDNA substrates listed in supplementary material and methods containing one hmU modification (hmU) or unmodified dsDNA (T). CPM (counts per minute), indicative of the transfer of glucose to DNA, were read for each sample. Experiment was performed in triplicate and error bars are representative of standard error. (B) UDP-Glo *in vitro* glucosyltransferase reaction. Recombinant JGT was incubated with 36nt long dsDNA substrates listed in Table 1 containing one hmU modification (hmU) or unmodified dsDNA (T). The amount of UDP Cleaved, indicative of the transfer of glucose to DNA, was estimated from a standard curve of UDP for each sample. Experiment was performed in triplicate and error bars are representative of standard error. (C) Representative substrate–velocity curve of recombinant JGT. Recombinant JGT activity with the 15nt long hmU-containing ds DNA substrate (WT substrate) listed in Table I. Glucosylation reactions were conducted with hmU DNA substrate concentrations of 0, 0.195, 0.391, 0.781, 1.563, 3.125, 6.25, 12.5, and 25 μM and fixed enzyme and UDP-glucose concentrations of 18 μM and 1 mM, respectively. Kinetic experiments were performed in triplicate and error bars are representative of standard error. UDP cleaved is plotted vs substrate concentration, and nonlinear regression was performed to determine kinetic parameters.

Table 1

Oligonucleotides used as substrates for glucosyltransferase assay*

Substrate	Sequence	kcat (s^{-1})	Km (μM)	kcat/Km (μM^{-1} s^{-1})
WT_2	5′-**TTAGGGTTAGGGTTA**-3′ 3′-**AATCCCAATCCCAAT**-5′	0.485 ± 0.022	0.519 ± 0.109	0.933
WT_1	5′-**TTAGGGTTAGGGTTA**-3′ 3′-**AATCCCAATCCCAAT**-5′	0.451 ± 0.025	0.523 ± 0.137	0.862
WT_3	5′-**TTAGGGTTAGGGTTA**-3′ 3′-**AATCCCAATCCCAAT**-5′	0.675 ± 0.037	0.519 ± 0.134	1.302
TTT	5′-**TTTGGGTTTGGGTTT**-3′ 3′-**AAACCCAAACCCAAA**-5′	0.703 ± 0.052	0.412 ± 0.149	1.705
ATT	5′-**ATTGGGATTGGGATT**-3′ 3′-**TAACCCTAACCCTAA**-5′	0.566 ± 0.034	0.267 ± 0.089	2.116
Random	5′-**GTACGAGTCGAGTCA**-3′ 3′-**CATGCTCAGCTCAGT**-5′	0.580 ± 0.021	0.313 ± 0.059	1.854

*Bold T indicates the position of an hmU modification.

**Glucosylation reactions were conducted with hmU DNA substrate concentration of 0, 0.195, 0.391, 0.781, 1.563, 3.125, 6.25, 12.5, and 25 μM and fixed enzyme and UDP-glucose concentrations of 18 μM and 1mM, respectively. UDP cleaved is plotted vs substrate concentration and nonlinear regression was performed to determine kinetic parameters.