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Epigenetic changes around the pX region and spontaneous HTLV-1 transcription are CTCF-independent

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RESEARCH ARTICLE

REVISED **Epigenetic changes around the pX region and spontaneous HTLV-1 transcription are CTCF-independent****[version 2; peer review: 2 approved]**

Previously titled: Spontaneous HTLV-1 transcription is accompanied by distinct epigenetic changes in the 5' and 3' long terminal repeats

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Any reports and responses or comments on the article can be found at the end of the article.

intrinsic pattern of epigenetic modifications that is independent of both the provirus insertion site and the chromatin architectural protein CTCF which binds to the HTLV-1 provirus.

Keywords

Histone modifications, DNA methylation, Epigenetics, Antisense transcription, Transcription kinetics, Retrovirus, HTLV-1, CTCF, CRISPR/Cas9, Single-molecule RNA-FISH

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REVISED Amendments from Version 1

In response to the reviewers' comments, we have carried out further experiments, and present these results in the revised manuscript, with minor alterations in the text. The major changes made are as follows:

- The title has been changed, following one reviewer's comment.
- Fifth paragraph in Introduction: Sentences and relevant references were inserted to refer to the effects on proviral transcription of p38 MAPK activation, polycomb repressive complex 1 (PRC1), HBZ mRNA, cell cycle and hypoxia.
- The terms "5' LTR" and "3' LTR" were systematically replaced with "5' LTR junction" and "3' LTR junction", respectively, and clarified at the beginning of the Results.
- ChIP-seq results (Figure 3, Figure 7a and Supplementary Figure 1b) were revised to present the reads within the LTRs. The section "High-throughput sequencing" in Methods was updated to clarify the procedure used to align the reads to an HTLV-1 reference genome.
- Results section: The observation on H3K4me3 in the Tax(+) and Tax(-) populations was described more in detail. A sentence was inserted, and the results shown in the revised and updated Supplementary Figure 1a.
- Results section: A phrase was inserted - "The DNA methylation pattern was much less variable in the HTLV-1-infected T cell clones than in the PBMCs: each clone is derived from a single cell, so every cell in that clone carries the HTLV-1 provirus in the same genomic site."
- Figure 9 was updated with data from a new experiment, and a section "Droplet digital PCR" was added to Results.
- The term "CTCF-mutant" was systematically replaced with "ΔCTCF-binding mutant".
- Figure 3, Figure 4, Figure 5, Figure 6, Figure 7, Figure 8, Figure 9 and corresponding figure legends were updated.
- Supplementary File 1, Supplementary Figure 1, Supplementary Figure 2 and Supplementary Figure 3 were updated.

See referee reports

Introduction

Human T cell leukemia virus type 1 (HTLV-1) was the first pathogenic exogenous retrovirus identified in humans. The main routes of infection are breast feeding, sexual contact and blood transfusion, each of which transmits cells carrying HTLV-1 and capable of infecting other cells in a new host. The majority of infected individuals remain asymptomatic throughout life. However some 5% develop adult T cell leukemia (ATL), and up to another 5% develop HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP)^{1,2}.

HTLV-1 reverse-transcribes its 9 kb genomic RNA into complementary double-stranded DNA which is then inserted into the host cellular DNA upon infection. Thereafter the virus remains as a chromatinized provirus and is replicated as a part of the host genome. The virus mainly resides in CD4⁺ T cells. Each infected cell carries a single copy of the HTLV-1 provirus in a given location in the host genome^{3,4}.

The provirus has identical long terminal repeats (LTRs) at the 5' and 3' ends, each of which serves as a promoter to drive the transcription of HTLV-1 from the plus- and minus-strand, respectively (Figure 1a) (reviewed in ref. 5). Most of the viral transcripts are from the plus strand: the transcripts yield a variety of viral proteins by alternative splicing. One of the plus-strand products is Tax, which has diverse functions in the infected cells including immortalization^{6,7}, accelerated cell cycle progression into S phase⁸, cell proliferation⁹ and DNA damage¹⁰. Tax also exerts a strong positive feedback on the promoter in the 5' LTR by assembling other transcription activators (reviewed in ref. 5). HTLV-1 encodes HBZ in the minus strand from the 3' LTR¹¹ (Figure 1a). This transcript also contributes to viral pathogenesis¹².

In HTLV-1-infected individuals, *tax* is often undetectable in freshly isolated peripheral blood mononuclear cells (PBMCs), whereas *HBZ* is almost invariably detectable¹³, revealing a sharp contrast between the plus- and minus-strand transcription. However, the evidence of constitutive reactivity of cytotoxic T cells (CTLs) against Tax *ex vivo*¹⁴ indicates that CTLs are frequently exposed to the viral antigen Tax *in vivo*, suggesting that *tax* is intermittently expressed *in vivo*. The current view of HTLV-1 expression *in vivo* is that *tax* is usually silenced but undergoes intermittent expression, whereas *HBZ* is almost constantly expressed, yet at a low level¹.

HTLV-1 expression in fresh, patient-derived PBMCs follows a characteristic trajectory. Once PBMCs are isolated from the blood of infected individuals and put into culture, a fraction of infected cells start expressing Tax within the first few hours¹⁵ (Figure 1b), perhaps triggered by the stress experienced by the cells on removal from the circulation¹⁶. Plus-strand bursts are triggered by cell stress via p38 MAPK activation and require deubiquitylation at the HTLV-1 promoter of histone H2A (Lys119), the inhibitory transcriptional mark characteristic of polycomb repressive complex 1 (PRC1)¹⁶. In patient-derived HTLV-1-infected clones *in vitro*, plus-strand bursts are more frequent in cells that lack *HBZ* mRNA and in cells in G0/G1 phase¹⁷, and more intense under conditions of physiological hypoxia¹⁸. In contrast, the abundance of *HBZ* mRNA remains relatively stable during short-term culture *in vitro* (Figure 1b).

In addition to examining fresh PBMCs, we investigated HTLV-1 transcription in HTLV-1-infected T cell clones established from patient-derived PBMCs⁴. These clones behave differently from the *ex vivo* PBMCs described above. We recently examined the plus- and minus-strand expression simultaneously at the single-cell level in these clones, by single-molecule RNA-FISH¹⁷. The plus strand shows a rapid and intense expression, often referred to as a burst, seemingly flanked by a period of transcriptional silence. In a clonal cell population, at any given time, a plus-strand burst is present in a fraction (5% to 30%) of cells, each cell containing hundreds of transcripts, leaving the other cells negative for the plus-strand expression (Figure 1c). On the other hand, *HBZ* is expressed relatively constantly, again providing evidence of asymmetric expression from the plus- and

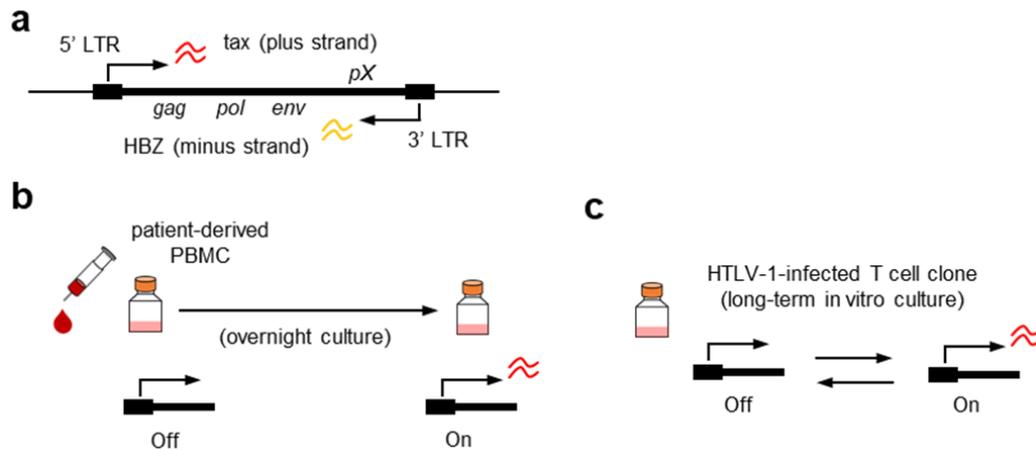


Figure 1. HTLV-1 transcription in two distinct models. **a)** Schematic diagram of HTLV-1 provirus inserted in the host genome. The HTLV-1 provirus has two identical LTRs, one at each end of the provirus. As well as genes encoding the canonical retroviral structural components Gag, Pol and Env, the provirus contains a group of regulatory genes in the pX region on the plus-strand. The plus-strand transcripts, represented by *tax*, are coloured in red, and the minus-strand transcript *HBZ* in yellow. **(b)** In PBMCs freshly isolated from HTLV-1 carriers, HTLV-1 reactivates and expresses the plus-strand transcripts within a few hours of culture; but these transcripts remain transcriptionally silent for most of the time *in vivo*. **(c)** In HTLV-1-infected T cell clones cultured *in vitro*, the promoter activity for plus-strand transcripts shuttles between the on and off state. The plus-strand transcripts are only produced when the promoter activity is on, yielding only a limited fraction of cells that are positive for the plus-strand transcripts at a given time.

minus-strand (Figure 1c). The simplest interpretation of these observations is that the kinetics of expression has reached equilibrium state at the population level, and that each cell in these HTLV-1-infected T cell clones continually switch on and off the 5' LTR promoter activity and so go through cycles of intermittent plus-strand expression.

We discovered that the host protein CTCF binds to the HTLV-1 provirus in the middle of the pX region¹⁹. CTCF is a chromatin-binding zinc-finger protein with a wide range of functions, including transcription regulation, insulation for repressive histone modifications, and chromatin looping. We postulated that the binding of CTCF regulates the epigenetic modifications in the provirus, and hence viral transcription.

In the present study we had two aims. First, to investigate the epigenetic modifications in the HTLV-1 provirus that accompany the dynamic changes in viral transcription during short-term culture of PBMCs, and in HTLV-1-infected T cell clones *in vitro*. Second, to examine the potential impact of CTCF on the epigenetic modifications and viral transcription. We altered the CTCF-binding site in the provirus with CRISPR/Cas9 technique to remove CTCF from the provirus, identified the epigenetic modifications and assayed viral transcription.

Methods

Cell culture

Peripheral blood mononuclear cells (PBMCs) from patients with the HTLV-1-associated inflammatory disease HAM/TSP were separated from peripheral blood with Histopaque (Sigma, H8889), washed in PBS, frozen in fetal bovine serum containing

10% DMSO and stored in liquid nitrogen until use. Upon thawing PBMCs, CD8⁺ cells were removed with Dynabeads (Invitrogen, 11147D). The cells were suspended (1×10^6 cells/ml) in RPMI-1640 supplemented with L-glutamine, penicillin/streptomycin and 10% fetal bovine serum, and incubated in 5% CO₂ at 37°C overnight.

HTLV-1-infected T cell clones⁴ were maintained in RPMI-1640 (Sigma, R0883) supplemented with L-glutamine, penicillin/streptomycin and 20% fetal bovine serum (Gibco, 10500-064) in 5% CO₂ at 37°C. IL-2 (Miltenyi Biotec, 130-097-745) was supplemented (100 unit/ml) into the culture twice a week. Raltegravir (Selleck Chemicals, MK-0518) was used at the concentration of 10 μM throughout the culture in order to prevent secondary infection.

Flow cytometry and cell sorting

PBMCs were stained for surface markers CD4 and CADM1 following LIVE/DEAD cell staining (Invitrogen, L34976). Then the viral protein Tax was stained intracellularly with Foxp3 staining kit (eBioscience, 00-5523-00) (Figure 2a). CADM1 staining was included in order to obtain an equivalent number of HTLV-1-infected cells in the Tax⁻ population²⁰. HTLV-1-infected T cell clones were stained with LIVE/DEAD and anti-Tax antibody (Figure 2b). The antibodies used were: mouse anti-CD4-PE (clone RPA-T4; BioLegend, 300507; concentration used, 0.8 μg/ml); chicken anti-CADM1-biotin (clone 3E1; MBL, CM004-6; 20 μg/ml) in combination with SA-BV421 (BioLegend, 405226; 1 μg/ml); mouse anti-Tax-Cy5 or anti-Tax-AF647 (clone LT-4; 0.4 μg/ml)²¹. Cell sorting was carried out with a BD FACSAria III.

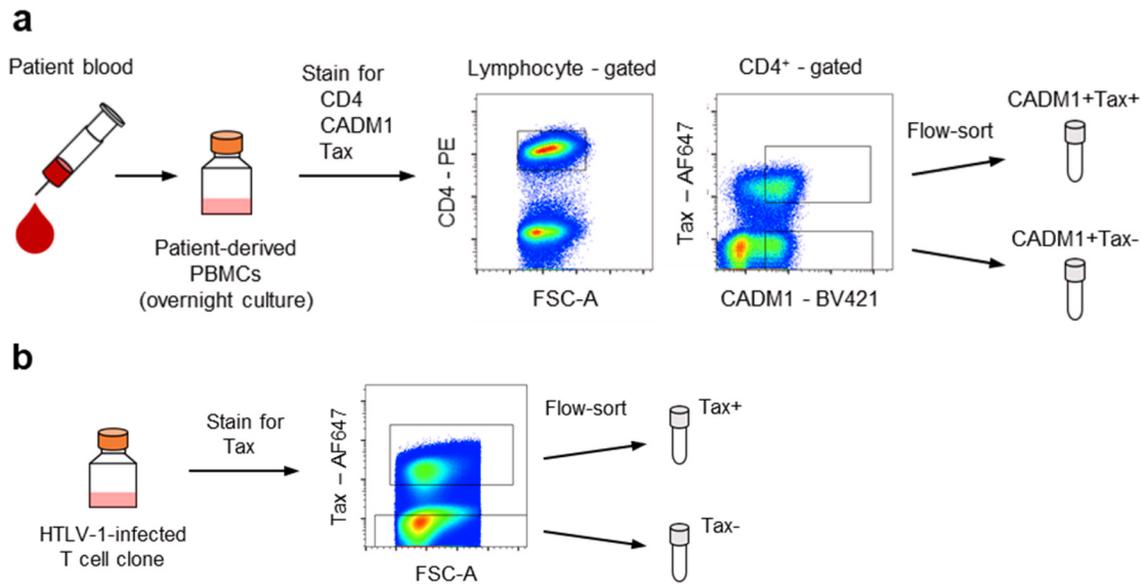


Figure 2. Overview of the cell preparations. (a) Preparation of Tax⁺ and Tax⁻ populations from PBMCs obtained from HTLV-1-infected patients. PBMCs were stained for CD4, CADM1 and Tax after overnight culture. Tax⁺ and Tax⁻ fractions were collected from the CADM1⁺ population. (b) Preparation of the Tax⁺ and Tax⁻ populations from HTLV-1-infected T cell clones. HTLV-1-infected T cell clones were stained for intracellular Tax and sorted according to Tax expression.

Chromatin immunoprecipitation

Chromatin was sonicated and sheared following cell and nuclear lysis. Sheared chromatin was incubated with each of the following antibodies: rabbit polyclonal anti-H3K4me3 (Millipore, 07-473; 2 µg per assay), rabbit polyclonal anti-H3K9Ac (Millipore, 17-658; 2 µg), mouse anti-H3K27Ac (clone CMA309; Millipore, 17-683; 2 µg), rabbit polyclonal anti-H3K36me3 (Abcam, ab9050; 2 µg), rabbit polyclonal anti-CTCF (Millipore, 07-729; 2 µg) and normal rabbit IgG (Millipore, PP64B attached to 17-658; 2 µg). The resulting immune complexes were precipitated with Protein A+G magnetic beads (Millipore, 16-663). After washing the beads, the DNA fragments were recovered and purified.

Library preparation and enrichment for the proviral DNA fragments

Adaptor DNA was attached to the fragments obtained from chromatin immunoprecipitation using NEBNext Ultra II DNA Library Prep Kit (NEB, E76455). Then DNA library fragments were enriched for the HTLV-1 proviral sequence by probe capture hybridization²². Namely, DNA fragments were incubated in hybridization buffer (NimbleGen, 05634261001) at 65°C for 4 hours with human Cot-1 DNA (Invitrogen, 15279-011) and biotinylated hybridization probes complementary to the HTLV-1 provirus sequence. After hybridization, the probes and associated DNA library fragments were recovered with Streptavidin beads (Invitrogen, 65305), and the DNA fragments were PCR-amplified using NEBNext High-Fidelity 2X PCR Master Mix (NEB, M0541) with primers P5 (5'-AAT GAT ACG GCG ACC ACC GA-3') and P7 (5'-CAA GCA GAA GAC GGC ATA CGA-3') to a concentration of the order of 10 nM (98°C for 45 sec; varying cycles of 98°C for 15sec, 65°C for 30 sec and 72°C for 30 sec; 72°C for 1 min).

High-throughput sequencing

The DNA library fragments were sequenced with Miseq Reagent Kit v3 (150 cycles) (Illumina).

All reads obtained from an HTLV-1 clone TBX4B were aligned to an HTLV-1 reference genome J02029 (ref. 23). The reads that overlapped either end of the provirus (J02029) were used to identify the host genomic sequence flanking the provirus in TBX4B (hg38 chr22: 43,927,318). The sequence around the provirus insertion site (TBX4B) was appended to the provirus (644 bp to the upstream and 632 bp to the downstream of the provirus sequence J02029) to create a custom reference for TBX4B. The J02029 reference was also used to align the reads obtained from PBMCs and other HTLV-1-infected T cell clones.

Paired 75 bp reads were aligned to the respective reference genome with BWA²⁴. Paired reads were kept if they were aligned within 80-800 nucleotides. PCR duplications were removed with Picard 2.6.0. The data was converted into the bedgraph format with bedtools²⁵, and visualised with the R Bioconductor package Sushi²⁶.

Methylated DNA immunoprecipitation

DNA was extracted from fixed and flow-sorted HTLV-1-infected cells with the DNA FFPE Tissue Kit (Qiagen, 56404). DNA was sheared by sonication (Covaris) to obtain 200-600 bp fragments. Fragments containing methylated DNA were precipitated using MethylCollector Ultra Kit (Active Motif, 55005) with Low Salt Binding Buffer supplied by the manufacturer. The fragments were purified from either precipitated or unbound fraction with MinElute PCR purification Kit (Qiagen, 28004), and eluted into the same volume. For each locus in the HTLV-1 provirus indicated in Figure 4a, the abundance of precipitated fragments relative to

the one from the unbound fraction was obtained by qPCR (95°C for 20 sec; 50 cycles of 95°C for 1 sec and 60°C for 20 sec) with the delta Ct method. The fraction size of captured fragments (%total) was calculated by $[B]/([B]+[U]) \times 100$, where [B] and [U] denote the relative abundance of precipitated and unbound fragments, respectively. PCR primers used are listed in [supplementary material \(Supplementary Table 1; Supplementary File 1\)](#).

Bisulfite treatment and DNA sequencing

DNA was extracted from fixed and flow-sorted HTLV-1-infected cells with the DNA FFPE Tissue Kit (Qiagen, 56404). Purified DNA was subject to bisulfite treatment with EpiTect Bisulfite Kit (Qiagen, 59104). A nested PCR was performed with FastStart Taq DNA Polymerase (Roche, 04738357001) to amplify the regions indicated in [Figure 5b](#). PCR conditions were as follows: 95°C for 5 min; 95°C for 30 sec, annealing temperature for 30 sec and 72°C for 30 sec (40 cycles); and 72°C for 2 min. The primer sequences and annealing temperatures are shown in the [supplementary material \(Supplementary Table 2; Supplementary File 1\)](#). The PCR products were cloned into pGEM-T Easy (Promega) and Sanger-sequenced (GATC Biotech).

CRISPR/Cas9 and cell cloning

Ribonucleoprotein (RNP) complex transfection was used²⁷. Namely, 2 µl of recombinant Cas9 protein (2.5 µg/µl) (PNA Bio, CP02) and 0.5 µl of *in vitro*-synthesised guide RNA (3 µg/µl) (Agilent, 5190-7706; DNA template, 5'-AAG CAC CGA CTC GGT GCC ACT TTT TCA AGT TGA TAA CGG ACT AGC CTT ATT TTA ACT TGC TAT GCT TTT CAG CAT AGC TCT AAA ACC GCG AGG TGG CGC TTT CTC CTA TAG TGA GTC GTA TTA CAT CG-3') as well as 1 µl of homologous DNA repair template (100 µM) (5'-AGG AAG CTG TGC TTG ACG GTT TGC TAT CCT TAG AAG AGG AAA GCC GCG GCC GGC TGC GAC GGG GCC CTC CAG GGG AGA AAG CCC CGC CAA GAG GTG AAA CGC ATC GTG ATC GGC AGC GAC GGG CTG AGG AGA AGA GGA AGC GAA AAA AAG AGC GGG AGA AAG AGG AGG AAA AGC AG-3') (Integrated DNA Technologies) were combined with 8 µl of R resuspension buffer from Neon transfection system (Invitrogen). Cells (5×10^5) were suspended in buffer containing RNP complex and transfection was performed with Neon (Invitrogen) (1600 V, 10 msec and 3 pulses). When cells had recovered at around a week after transfection, DNA was extracted with DNA extraction solution (Epicentre, QE09050). The absolute copy numbers of *gag* and the mutant proviral sequence of the CTCF site in the DNA were quantified by qPCR (50°C for 2 min; 95°C for 10 min; 50 cycles of 95°C for 15 sec and 60°C for 1 min) with respective standard curves, in order to estimate the frequency of mutant cells in the culture. Primers and probes for *gag* (forward, 5'-TTA TGC AGA CCA TCC GGC TT-3'; reverse, 5'-TAT CTA GCT GCT GGT GAT GGA G-3'; probe, 5'-CGG TGC AGC AGT TTG ACC CCA CTG C-3') and mutant CTCF-binding site (forward, 5'-CTG CTT TCT CCG GGC GAC or CTG CTT TCT CCG GGC AAA G-3'; reverse, 5'-AGC CCC GCC AAG AGG T-3'; probe, 5'-AAC GCA TCG TGA TCG GCA GCG AC-3') were used. Mutants were detected at a frequency of 1.6% to 3.1% in each of 4 HTLV-1-infected T cell clones applied. Cells were subcloned in order to isolate mutant cells by either limiting dilution or

flow-sorting (BD FACSAria III) under Containment Level 3 conditions. Subclones were screened for the mutant proviral sequence of the CTCF site by PCR (95°C for 20 sec; 40 cycles of 95°C for 1 sec and 60°C for 20 sec) with a mutation-specific primer indicated above. The DNA sequence of the putative mutants was confirmed by Sanger sequencing (GATC Biotech).

Single-molecule RNA-FISH

HTLV-1-infected T cell clones were subjected to single-molecule RNA-FISH, targeting the plus- or minus-strand transcripts of HTLV-1, following the protocol described previously¹⁷. The coverslips were imaged with an Olympus IX70 inverted widefield microscope with a 100× 1.35NA UPlanApo oil objective lens, a Spectra Light Engine illumination source (Lumencor) and an ORCA-Flash 4.0 V2 digital CMOS camera (Hamamatsu).

Droplet digital PCR

The DNA samples obtained by ChIP using anti-CTCF antibody or normal rabbit IgG described above were quantified by droplet digital PCR. The reaction was set up using ddPCR Supermix (Bio-Rad, 186-3023) containing 750 nM each of forward and reverse primers and 250 nM probe to detect the HTLV-1 CTCF-binding site and TC-1 locus. Primers and probe for the HTLV-1 CTCF-binding site are listed in [Supplementary File 1](#) (Target pX in Table 1). For TC-1 locus: forward primer, 5'-TCT CCA GCA CTT CTT GCT CA-3'; reverse primer, 5'-TGG GAT GGC TAA CCT GTT GT-3'; probe, 5'-TCT CTG CTG CTC CCA GGC GGC-3'. Droplets were generated with QX200 Droplet Generator (Bio-Rad). PCR was performed (95°C for 10 min; 40 cycles of 94°C for 30 sec and 60°C for 1 min; 98°C for 10 min) with C1000 Touch thermal cycler (Bio-Rad). Fluorescent signal was detected using QX200 Droplet Reader (Bio-Rad).

Results

To identify the epigenetic modifications associated with transcriptional activity in the provirus, we sorted the cells based on Tax protein expression and performed ChIP and DNA methylation analyses for each fraction ([Figure 2](#)) unless stated otherwise.

In the descriptions of the ChIP-seq data, we use the terms “5' LTR junction” and “3' LTR junction” to denote the regions of the HTLV-1 provirus adjoining the 5' LTR and the 3' LTR, respectively.

Histone modifications are strongly associated with plus-strand transcription

We first studied *in vitro* HTLV-1-infected T cell clones, because the cells in each clone share the same provirus insertion site, so minimizing effects due to heterogeneity in the host genomic environment of the provirus. We performed ChIP on the Tax⁺ and Tax⁻ populations from one of the HTLV-1-infected T cell clones to identify the histone modifications in the HTLV-1 provirus ([Figure 3a](#)). The HTLV-1 provirus was marked with H3K4me3 from the 5' LTR junction through to the 3' LTR junction in the Tax⁺ population. Substantial signals from other histone marks H3K9Ac and H3K27Ac were also detected in the 5' LTR junction and *gag* in the Tax⁺ population. These histone marks

are associated with promoters and enhancers with active transcription²⁸. In the pX region and the 3' LTR junction, these three histone marks (H3K4me3, H3K9Ac and H3K27Ac) were constantly detected, regardless of Tax expression. In particular, H3K4me3 was more highly enriched in those regions in the Tax⁻ population (Figure 3a and Supplementary Figure 1a), consistent with the observation of differential expression of the plus- and minus-strands. A similar pattern was observed in another *in vitro* HTLV-1-infected T cell clone (11.65)

(Supplementary Figure 1b). The small signal of H3K4me3 in the Tax⁻ population (Figure 3a and Supplementary Figure 1b) is a characteristic feature of a poised promoter.

Next, we examined patient-derived PBMCs (patient TW for H3K4me3 and H3K36me3; patient TCD for H3K9Ac and H3K27Ac) after overnight culture (Figure 3b). The pattern of histone modifications was largely the same as that observed in HTLV-1 clones, in that there was a much stronger signal for

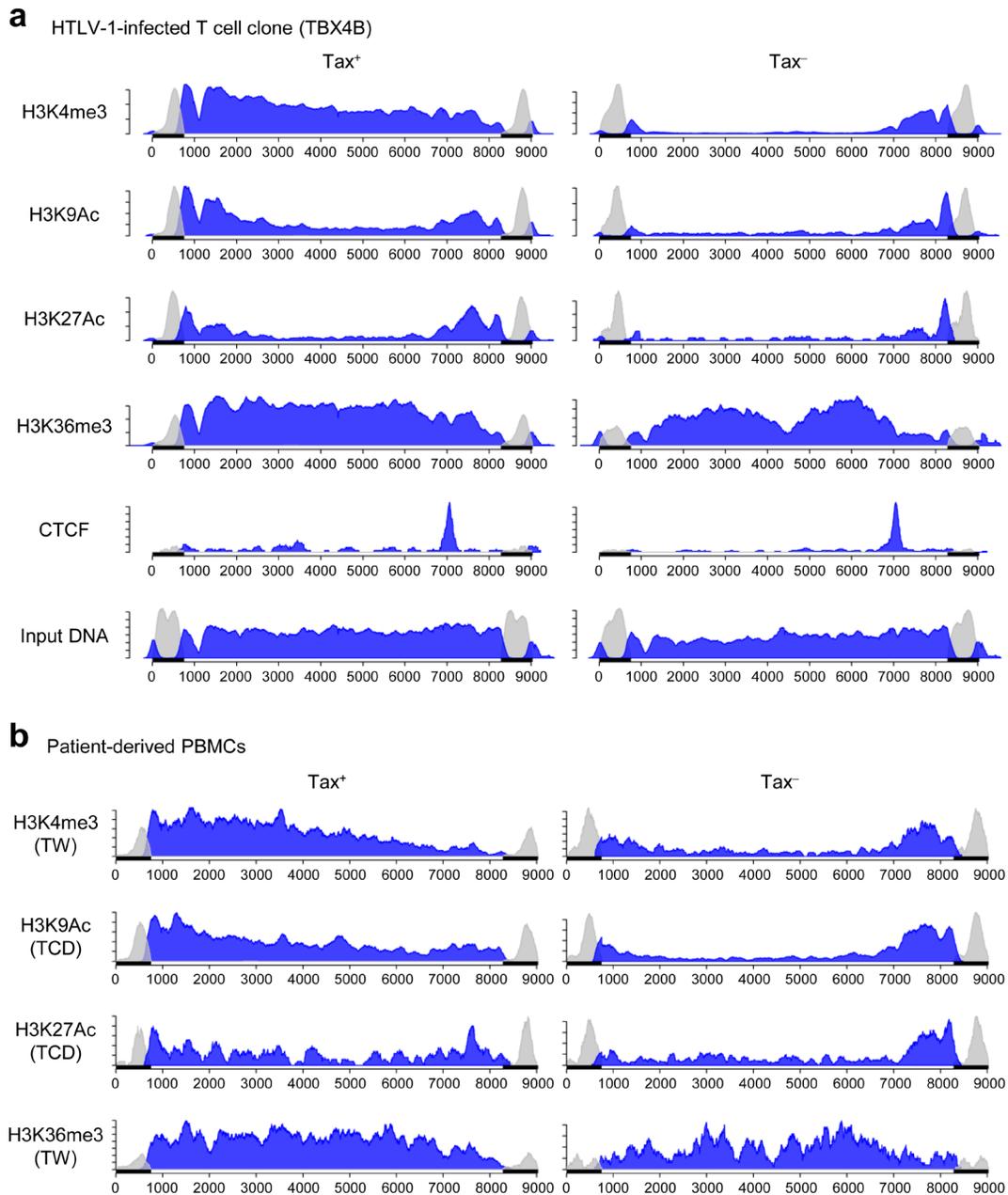


Figure 3. Histone modifications and CTCF-binding in the HTLV-1 provirus. Chromatin immunoprecipitation (ChIP) was used to identify (a) histone modifications and CTCF-binding in the Tax⁺ and Tax⁻ populations from an HTLV-1-infected T cell clone (TBX4B); and (b) histone modifications in the CADM1⁺Tax⁺ and CADM1⁺Tax⁻ populations from PBMCs obtained from HTLV-1-infected patients (Patients TW and TCD). The horizontal axis indicates the nucleotide position in the full-length HTLV-1 provirus (J02029), and the vertical axis the read depth (arbitrary units). The reads that aligned within either one of the LTRs are greyed out. The black bars on the horizontal axis indicates the LTRs.

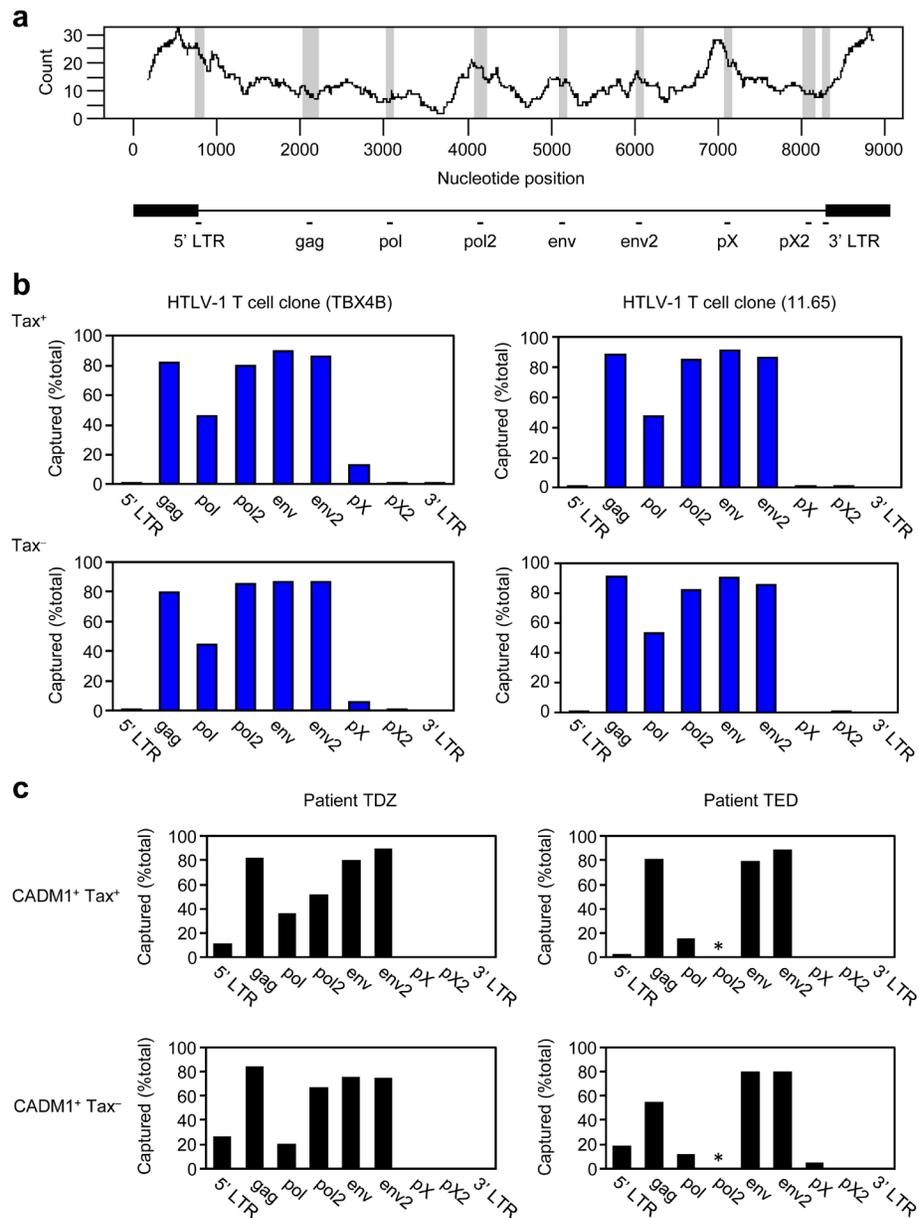


Figure 4. DNA methylation across the body of the HTLV-1 provirus. (a) Upper panel: count of CpG dinucleotides in a window of 350 bp in the HTLV-1 reference genome (L36905). Lower panel: schematic diagram of HTLV-1 provirus indicating the two LTRs and the 9 loci examined by MeDIP. (b) DNA methylation on the HTLV-1 provirus in the Tax⁺ and Tax⁻ populations from two HTLV-1-infected T cell clones (Clones TBX4B and 11.65). (c) DNA methylation on the HTLV-1 provirus in the CADM1⁺Tax⁺ and CADM1⁺Tax⁻ populations in PBMCs from two unrelated individuals (Patients TDZ and TED). The asterisk (*) indicates that the PCR failed to amplify.

H3K4me3, H3K9Ac and H3K27Ac from the 5' LTR junction in the Tax⁺ population, and those marks appeared persistent in the 3' LTR junction regardless of viral reactivation.

Because we observed a similar pattern of histone marks in two independent HTLV-1-infected T cell clones with distinct provirus insertion sites, as well as in PBMCs with polyclonal insertion sites, the pattern of histone marks that we observed is not likely to be dependent on the host genomic environment of

the provirus, but rather is a feature intrinsic to the HTLV-1 provirus. Two observations indicate that the changes in the observed pattern of histone marks are rapid and reversible: first, HTLV-1 reactivation takes place within a few hours of culture in patient-derived PBMCs^{15,16}; second, the HTLV-1-infected T cell clones demonstrate intermittent bursts of Tax expression^{17,29}. Currently, there is no means of separating HBZ⁺ and HBZ⁻ populations. However, we anticipate that the changes in histone marks in the 3' LTR junction are likely to be small,

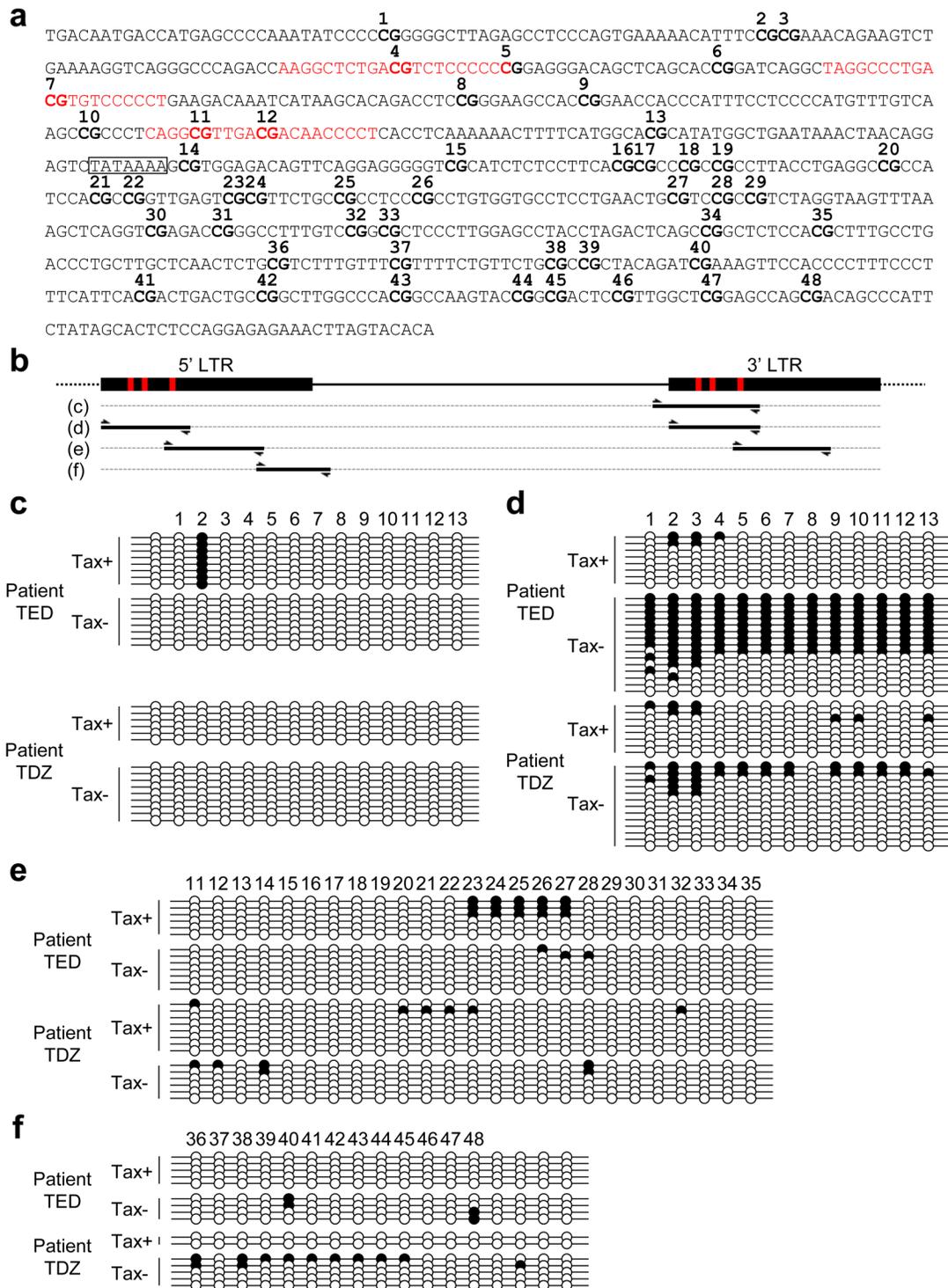


Figure 5. DNA methylation in the HTLV-1 LTR of patient-derived PBMCs. (a) The HTLV-1 LTR sequence (Accession no. L36905) with CpG dinucleotides highlighted in bold. The three TREs are coloured in red; the TATA box is indicated in the rectangle. (b) Schematic diagram of HTLV-1 provirus and the regions amplified with indicated sets of primers for bisulfite-sequencing. Sequencing results for each region are shown in the corresponding panels (c-f). The three TREs are indicated by red bars. (c-f) Schematic representation of DNA methylation for each clone sequenced. Open circles indicate unmethylated cytosine; closed circles indicate methylated cytosine. The numbers indicate the corresponding CpG sites in panel (a).

and the minus-strand expression is much more constant than the plus-strand transcription¹⁷.

Confirming our previous report¹⁹, we observed binding of the host protein CTCF in the HTLV-1 provirus (Figure 3a) at the boundary in the pX region where the histone marks H3K4me3, H3K9Ac and H3K27Ac are confined to the 3' end of the provirus. We hypothesized that the host protein CTCF regulates the epigenetic modifications: we discuss this hypothesis below.

DNA in the pX and 3' LTR region is left unmethylated regardless of the plus-strand expression

Next we wished to examine if DNA methylation in the provirus also correlates with HTLV-1 proviral transcription, as we saw in the histone marks above. We performed methylated DNA immunoprecipitation (MeDIP) and examined 9 loci across the HTLV-1 provirus by qPCR (Figure 4a). In the HTLV-1-infected T cell clones (TBX4B and 11.65) (Figure 4b), regardless of Tax expression, DNA in the HTLV-1 provirus was methylated in the *gag*, *pol* and *env* regions, whereas the pX and 3' LTR regions were not methylated. The signal from the *pol* locus was lower than the other sites, perhaps because there are fewer CpG sites in this locus (Figure 4a). Note that, on the contrary, the pX region was hypomethylated (Figure 4b) despite the higher frequency of CpG in this region (Figure 4a).

We also examined patient-derived PBMCs (Patients TDZ and TED) (Figure 4c) cultured overnight. Regardless of the plus-strand reactivation, DNA in the region from *gag* to *env* was heavily methylated. On the other hand, again, the pX and 3' LTR regions were not methylated. The *pol2* site in Patient TED was not detected, perhaps because of sequence polymorphism in HTLV-1.

Regardless of the plus-strand expression, the pattern of DNA methylation in the body of the HTLV-1 provirus was essentially the same: that is, largely methylated but for the pX and 3' LTR regions. The CTCF-binding site in the HTLV-1 provirus is in the pX region (Figure 3a): as previously reported¹⁹, this CTCF-binding site is situated at the observed border of DNA methylation (Figure 4b and c).

DNA hypomethylation in the plus-strand promoter is a prerequisite for viral reactivation

To investigate further the putative link between the DNA methylation and viral expression, we examined the HTLV-1 plus-strand promoter region. In the first half of the HTLV-1 LTR, there are three Tax-response elements (TREs), among other transcription factor binding sites, upstream of the TATA box (Figure 5a). The TREs serve as the promoter for plus-strand transcription. We used fresh, patient-derived PBMCs, as they are most likely to maintain the DNA methylation pattern *in vivo*. There are 48 CpG sites in the HTLV-1 LTR (Accession number L36905) (Figure 5a). Because the two HTLV-1 LTRs have an identical sequence, it is not possible to specifically amplify one of the LTRs for bisulfite-sequencing. Therefore we took the approach of Koiwa *et al.*³⁰, as follows.

First, the 5' half of the 3' LTR was specifically amplified as is indicated in Figure 5b by line (c). None of the fragments were methylated, except for position 2 in the Tax⁺ population from Patient TED (Figure 5c). (Here, we designate fragments with a few positions methylated as hypomethylated.) This observation was consistent with what we observed in the MeDIP assay targeting the 3' LTR (Figure 4c). Next, the same part of the HTLV-1 LTR was amplified from either the 5' LTR or 3' LTR, as is indicated by line (d) in Figure 5b. We assume that fragments are amplified from the 5' LTR and 3' LTR with equal efficiency. The results (Figure 5c) showed that most fragments from the 3' LTR were hypomethylated. DNA methylation in the three TREs (position 4–12) was observed exclusively in the Tax⁻ population (Figure 5d). We infer that, in most of the Tax⁻ cells in Patient TED, the 5' LTR promoter is methylated. In the Tax⁻ population from Patient TDZ, DNA methylation in the 5' LTR appeared less frequent, which leaves open the possibility that the DNA methylation is not the determining factor for viral latency. On the other hand, the 5' LTR promoter was likely to be hypomethylated when Tax was expressed in both patients. Then we expanded the search area for the DNA methylation further into the LTR, as indicated by lines (e) and (f) (Figure 5b). In general, CpG sites after position 13, and hence downstream of the TREs, were not heavily methylated whether in the 5' LTR or 3' LTR, and regardless of Tax expression (Figure 5e). Similarly, the 3' end of the 5' LTR was not methylated (Figure 5f), with an apparent exception in the Tax⁻ population from Patient TDZ.

To summarise, DNA methylation in the HTLV-1 LTR is confined within the first half of the LTR, which contains three TREs. DNA is not methylated when the plus strand is expressed; however, it is not yet clear whether DNA methylation alone is sufficient to explain proviral latency. In HTLV-1-infected T cell clones cultured *in vitro*, the 5' LTR promoter was hardly methylated, regardless of whether Tax is expressed at a given time (Supplementary Figure 2a and b). The DNA methylation pattern was much less variable in the HTLV-1-infected T cell clones than in the PBMCs: each clone is derived from a single cell, so every cell in that clone carries the HTLV-1 provirus in the same genomic site. Nevertheless, this reinforces the notion that DNA methylation is not the sole factor that suppresses, even if temporarily, the viral transcription.

Altering the sequence of CTCF-binding site in the HTLV-1 provirus by Cas9/gRNA RNP transfection

We observed above that the CTCF-binding site in the HTLV-1 provirus coincides with the apparent boundary of epigenetic modifications (i.e. both the histone marks and DNA methylation) in the provirus. Considering the known functions of CTCF, which include transcription regulation and the formation of an epigenetic barrier, we hypothesized that CTCF in the pX region of the HTLV-1 provirus controls the epigenetic modifications and viral transcription. To test this hypothesis, we applied CRISPR/Cas9 modification to alter the sequence of the CTCF-binding site, using ribonucleoprotein complex transfection²⁷. We isolated mutant cells by subcloning and confirmed that the

sequence at the CTCF-binding site had been correctly altered (Figure 6a). We previously showed that this alteration is sufficient to abrogate CTCF binding to the provirus¹⁹. Nevertheless, we observed that the mutant clones still expressed Tax (Figure 6b). We therefore set out to examine the epigenetic modifications in Δ CTCF-binding clones as described below.

The epigenetic modifications in the HTLV-1 provirus are CTCF-independent

We examined the epigenetic modifications in a Δ CTCF-binding clone (TBX4B-78). First, we confirmed that CTCF was no longer detected in either the Tax⁺ or Tax⁻ populations (Figure 7a). The pattern of the histone marks (Figure 7a) was largely the same as that in the parental clone (Figure 3a), in that the changes in H3K4me3, H3K9Ac and H3K27Ac were associated with Tax expression, and were stable downstream of nucleotide ~7000 (CTCF-binding site). The profile of H3K36me3 showed a reproducible small dip in the middle of the provirus specifically in the Tax⁻ cells. Similarly, DNA methylation in the body of the provirus (Figure 7b) was not distinct from that in the parental clone (Figure 4b): *gag*, *pol* and *env* were largely methylated, whereas the pX and 3' LTR regions were not (again downstream of the CTCF-binding site). The 5' LTR promoter was also hypomethylated, regardless of Tax expression in Δ CTCF-binding clones (Supplementary Figure 2a and c). These results are inconsistent with the idea that CTCF imposes an epigenetic border in the HTLV-1 provirus and regulates the distinct modifications around the pX region.

Since the technique of ChIP-sequencing coupled with probe capture hybridization lacks precise quantification, we went on to quantify the kinetics of the plus- and minus-strand transcription of HTLV-1 more precisely, using our previously described protocol of single-molecule RNA-FISH, to detect any possible impact of CTCF-binding on HTLV-1 transcription.

Removal of CTCF does not affect the kinetics of the plus- and minus-strand expression *in vitro*

We have recently reported that the HTLV-1 plus-strand transcription shows periods of transient, rapid and intense spontaneous activity, often referred to as a transcriptional burst, whereas the minus-strand expression is relatively stable¹⁷. To test whether CTCF has an impact on the pattern of the plus- and minus-strand expression, we performed single-molecule RNA-FISH on the

Δ CTCF-binding clones. Representative images are shown in Figure 8a. As reported in our recent publication¹⁷, a limited fraction of cells had a large number of plus-strand transcripts, and the remaining cells were negative. On the other hand, minus-strand transcripts were present in most cells. The number of transcripts per cell in the Δ CTCF-binding clones is presented in Figure 8b. The distribution of the plus-strand transcripts was indistinguishable between the Δ CTCF-binding and unmodified subclones from TBX4B. This trend was also the case for the minus-strand transcript (*HBZ*). We confirmed this result with another HTLV-1-infected clone (11.50) (Supplementary Figure 3). This result shows that the removal of CTCF did not affect the transcriptional activity of HTLV-1. Therefore, it is unlikely that any difference in the degree of epigenetic modifications between the CTCF-mutant and parental HTLV-1-infected T cell clones has a significant impact on the transcriptional activity of HTLV-1.

CTCF occupancy does not predict the viral reactivation in patient-derived PBMCs

The putative impact of CTCF in the provirus was tested above on the HTLV-1-infected T cell clones. These clones were initially isolated and expanded from PBMCs of HTLV-1-infected individuals, and show robust growth *in vitro*. It is likely that they were selected for strong *in vitro* growth, and so may have diverged phenotypically from the PBMCs *in vivo*; such differences could conceivably affect HTLV-1 transcription. Therefore, we wished to test the putative association between CTCF-binding and viral reactivation using PBMCs from HTLV-1-infected subjects.

It is estimated³¹ that there are tens of thousands of different HTLV-1-infected T cell clones in a typical HTLV-1-infected individual. Each clone carries a single copy of the provirus inserted in a unique location in the genome. Whether the viral reactivation takes place has a strong dependence on the genomic insertion site of the provirus³². We asked if those clones that reactivate the plus-strand transcription have differential CTCF occupancy in the provirus from those that remain silent after the short-term culture. We performed a ChIP assay targeting CTCF on the Tax⁺ and Tax⁻ populations after overnight culture, and performed droplet digital PCR to quantify the CTCF occupancy. The results showed no measurable difference in CTCF-binding between the two populations (Figure 9).

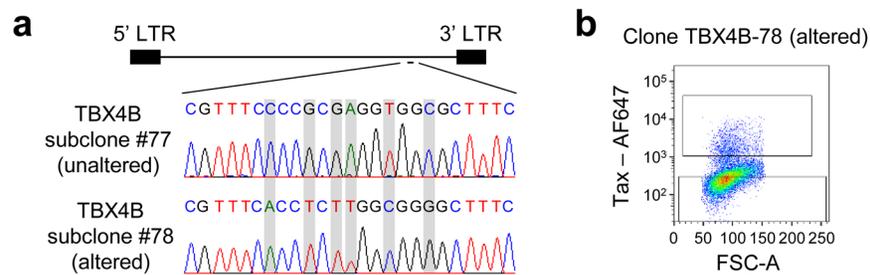


Figure 6. Alteration of the CTCF-binding site in the provirus in HTLV-1-infected T cell clones. (a) The sequence of the CTCF-binding site in the HTLV-1 provirus. The upper panel is from a subclone with the sequence unchanged, and the lower panel from a subclone in which the sequence was altered by CRISPR/Cas9 modification. (b) Flow cytometric analysis of the mutated clone after staining for intracellular Tax protein.

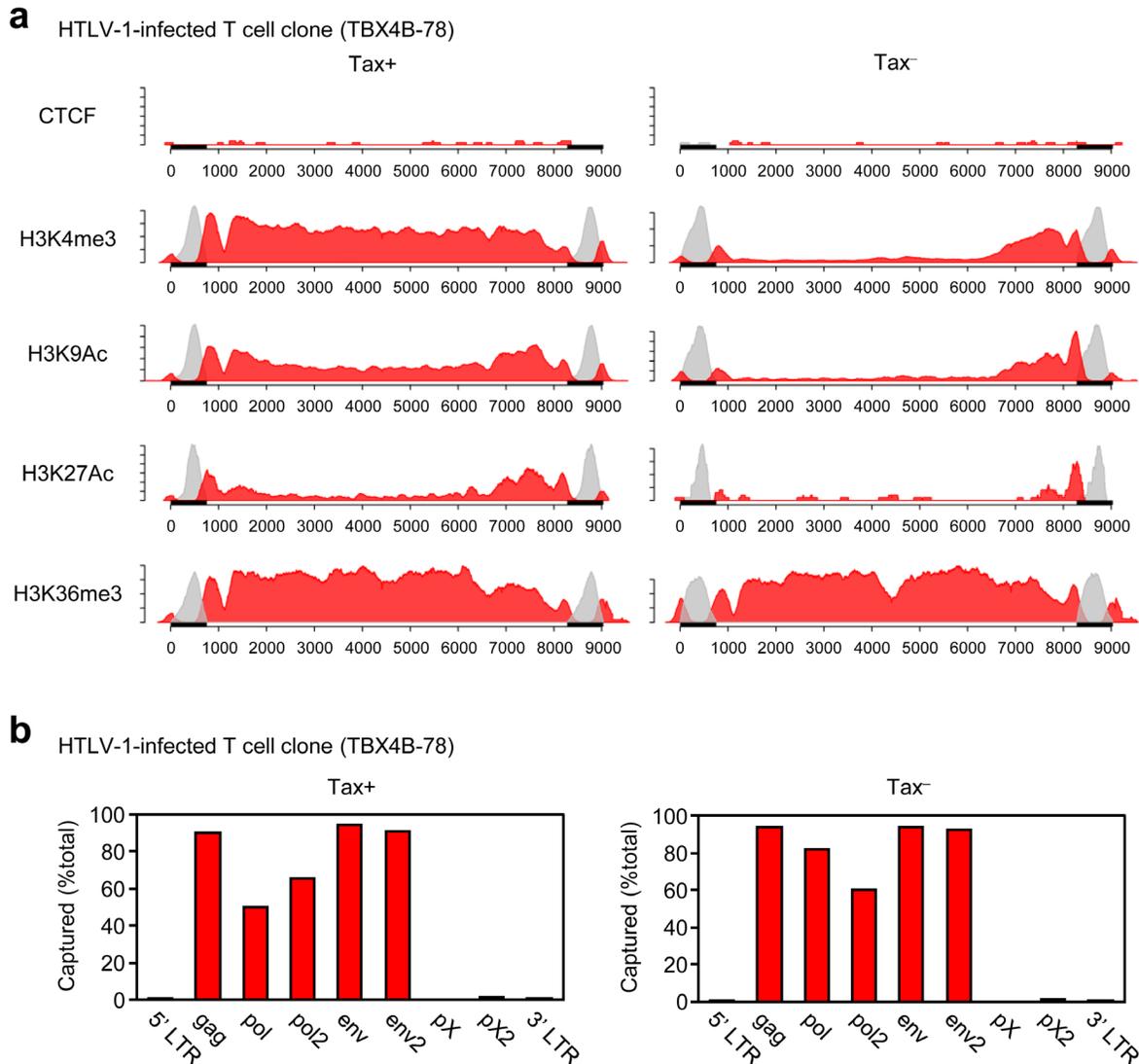


Figure 7. Epigenetic modifications in the HTLV-1 provirus lacking CTCF binding. (a) Histone modifications in the Tax⁺ and Tax⁻ populations of the altered HTLV-1-infected T cell clone (Subclone #78 of TBX4B). (b) DNA methylation in the body of the provirus in TBX4B-78. Note the similarity to the profiles of epigenetic modifications in the wild-type TBX4B (Figure 3).

Discussion

HTLV-1 has two identical LTRs, one at each end of the provirus, which serve as the promoters for the plus- and minus-strand transcription. One of the conundrums is how HTLV-1 keeps the plus-strand predominantly silenced while sparing the minus-strand expression *in vivo*. Attempts have been made to investigate the epigenetic modifications that differ between the 5' LTR and 3' LTR^{19,30,33,34}. However, recent work by us¹⁷ and others²⁹ shows that the HTLV-1 plus-strand transcription is highly variable over time. Thus, the epigenetic modifications we observe are an average of the two populations: one that is actively transcribing the plus strand, and the remaining cells that are not transcribing the plus strand at that time. Therefore in this study, we investigated what epigenetic

modifications accompany the plus-strand transcription, by separating the two HTLV-1-infected populations.

Our results show that the changes in histone marks H3K4me3, H3K9Ac and H3K27Ac in PBMCs are specific to the Tax⁺ population (Figure 3b). These histone marks are generally accompanied by active transcription, which in the case of HTLV-1 begins within the first few hours of culturing PBMCs^{15,16}. In *in vitro* HTLV-1-infected T cell clones, proviral transcription is not a one-off event: each cell appears to switch on and off the plus-strand transcription¹⁸. We captured a snapshot of histone modifications when Tax is expressed (Figure 3a). We conclude that the changes in histone modifications in HTLV-1 are highly dynamic: they are rapid and reversible.

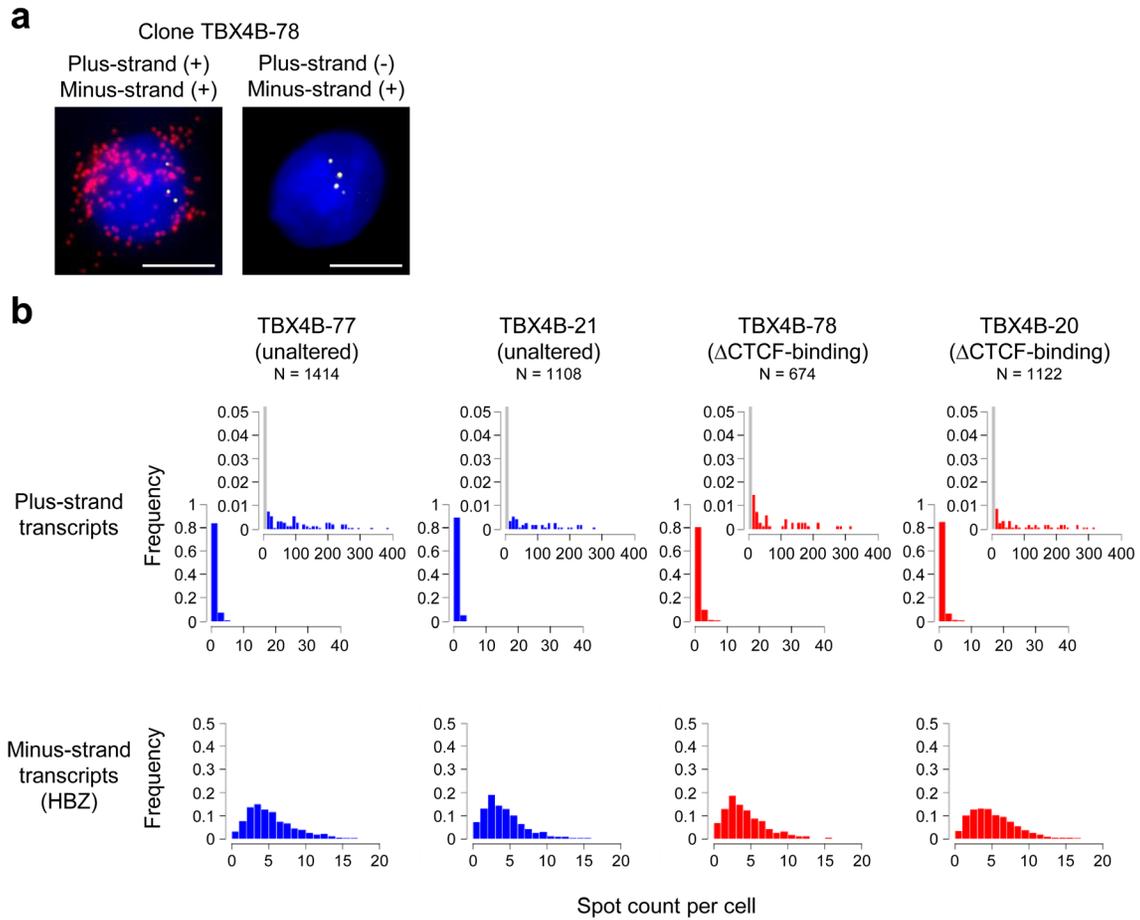


Figure 8. Kinetics of the plus- and minus-strand transcription in HTLV-1-infected T cell clones. (a) Representative images of HTLV-1 transcripts by single-molecule RNA-FISH (maximum-projection of Z-stacks). Red spots indicate the plus-strand transcripts, and yellow spots the minus-strand transcripts. Blue indicates the DAPI-stained nucleus. Plus- and minus-signs in brackets indicate respectively the presence or absence of the mRNA. Scale bar (white) = 5 μ m. (b) Spot counts of the plus-strand (upper row) and the minus-strand transcripts (lower row) respectively in the unaltered and Δ CTCF-binding subclones. The insets in the upper row capture low-frequency events on a magnified y-axis. The bar in the first bin in the insets is greyed out because it is out of scale.

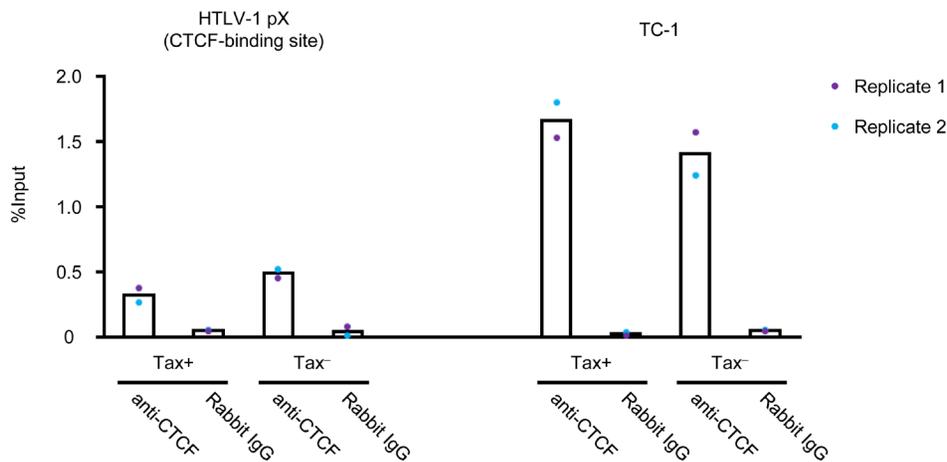


Figure 9. CTCF occupancy in the HTLV-1 provirus in patient-derived PBMCs. CTCF occupancy was examined by droplet digital PCR following ChIP for CTCF. The experiment was carried out on PBMCs after overnight incubation *in vitro*. Replicate 1 is obtained from pooled samples of 4 patients (TCR, TEJ, TED and TW) and Replicate 2 from 3 patients (TED, TCR and TEJ).

DNA in the body of the HTLV-1 provirus is largely methylated except for the pX region and 3' LTR³⁴. DNA methylation in the *gag*, *pol* and *env* regions has been considered as one of the mechanisms by which HTLV-1 maintains latency³⁴. However, in this study, we observed DNA methylation in the body of the provirus even in the Tax⁺ population (Figure 4c). Therefore, we conclude that DNA methylation in the gene body of the provirus has little impact on HTLV-1 transcription. HTLV-1 rapidly reactivates in *ex vivo* culture, but it is not yet known whether this is a physiological response or whether it results from a supraphysiological stress. It is possible, although we consider it unlikely, that the DNA methylation in the body of the provirus helps to maintain HTLV-1 latency *in vivo*.

DNA hypomethylation in the 5' LTR promoter is associated with proviral transcription, as previously reported³⁰. We conclude that DNA hypomethylation in the plus-strand promoter is likely to be required for viral reactivation, consistent with the idea³⁰ that DNA methylation preferentially silences plus-strand expression *in vivo*. However, it is not proved whether DNA methylation is sufficient for HTLV-1 latency, because our results do not permit an accurate estimate of the frequency of methylation in the Tax⁻ population. High-throughput sequencing for bisulfite-treated DNA is required to reveal the accurate view of DNA methylation associated with HTLV-1 latency. Nevertheless, assuming that DNA methylation is stable during the short-term culture of HTLV-1³⁵, we propose the following: (1) there are two categories of HTLV-1-infected cells *in vivo* - those with the 5' LTR promoter methylated and those in which it is hypomethylated; (2) viral reactivation is allowed only in the hypomethylated cells; and (3) the DNA methylation in the body of the provirus does not influence viral reactivation *ex vivo*. Whether proviral reactivation takes place depends strongly on the provirus insertion site³². It is therefore possible that DNA methylation in the 5' LTR promoter is related to the provirus insertion site.

Following the discovery that HTLV-1 binds CTCF¹⁹, we hypothesized that CTCF imposes a boundary in the histone modifications and DNA methylation around the CTCF-binding site in the pX region. However, our results do not support the hypothesis that the pattern of epigenetic modifications in HTLV-1 depends directly on CTCF-binding. It is possible that changes in epigenetic modifications would take a longer time to become established after the provirus is mutated, or that CTCF imposes an epigenetic boundary at the pX region in the early stage of infection and becomes dispensable thereafter. However, the chromatin boundary in the pX region is not static: our observations indicate that, each time a cell goes through the cycle of plus-strand expression, the histone modifications change rapidly in the provirus, yet they always return to the marks present in the previous state of plus-strand expression, even without CTCF binding. We conclude that CTCF binding does not directly impose a barrier to the spread of these epigenetic modifications. Instead, it is possible that CTCF confers a benefit on HTLV-1 by making chromatin loops with the nearby host genome³⁶. However, the consequences of HTLV-1 inserting an ectopic CTCF-binding site in the host genome vary widely according to the genomic integration site: it remains to be seen

whether there is an additional impact of CTCF binding to the HTLV-1 provirus that is consistent in all clones. It is also possible that CTCF confers a higher rate of HTLV-1 transmission and increases viral persistence *in vivo*. However, long-term animal model experiments may be necessary to test this hypothesis. It remains an open question what regulates the distinct epigenetic modifications observed around the pX region in HTLV-1.

Data availability

The following datasets are available from Open Science Framework:

Dataset 1 (ChIP-seq on epigenetic marks):
<https://doi.org/10.17605/OSF.IO/4Q9RY>³⁷

Dataset 2 (image data on smFISH):
<https://doi.org/10.17605/OSF.IO/4TBQY>³⁸

Dataset 3 (qPCR and ddPCR data from ChIP and MeDIP):
<https://doi.org/10.17605/OSF.IO/8M76K>³⁹

Dataset 4 (Sanger sequencing data from bisulfite assay):
<https://doi.org/10.17605/OSF.IO/D62H7>⁴⁰

These datasets are available under a CC0 1.0 Universal licence.

Consent

Human subjects: All donors gave written informed consent in accordance with the Declaration of Helsinki to donate blood samples to the Communicable Diseases Research Tissue Bank, approved by the UK National Research Ethics Service (15/SC/0089).

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Supplementary material

Supplementary Figure 1: Histone modifications in HTLV-1 provirus

[Click here to access the data](#)

Supplementary Figure 2: DNA methylation in the 5' LTR promoter in HTLV-1-infected clones

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Supplementary Figure 3: Count of viral transcripts in an HTLV-1+ T cell clone

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Supplementary File 1: File containing the following supplementary tables listing primers and probes used for PCR amplification.

[Click here to access the data](#)

Supplementary Table 1: PCR primers used for methylated DNA immunoprecipitation and qPCR.

Supplementary Table 2: PCR primers and annealing temperature to amplify bisulfite-treated HTLV-1 provirus.

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Paul M Lieberman

Gene Expression & Regulation Program, The Wistar Institute, Philadelphia, PA, USA

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 14 September 2018

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Vincenzo Ciminale

Department of Surgery, Oncology and Gastroenterology, University of Padua, Padua, Italy

In the present study Miura et al., carried out a study aimed at investigating the epigenetic changes in the HTLV-1 genome that take place upon (re)activation of viral transcription.

Based on their findings the authors conclude that the histone modifications H3K4me3, H3K9Ac and H3K27Ac correlated with plus-strand expression. Furthermore, plus-strand transcription was associated to promoter hypomethylation. This pattern of viral epigenetic modifications appeared to be independent of both the provirus insertion site and the binding of the CTCF chromatin architectural protein.

The paper is clearly written and the pertinent literature is appropriately cited and discussed.

The experiments are well laid-out and technically sound. The conclusions are well supported by the results and address an important aspect of the HTLV-1 life cycle. The methods are well described and appropriate.

As the T-cell clones show a mixture of Tax positive and Tax-negative cells, it would be very interesting to synchronize viral expression in these cells (e.g. by cell cycle double block or by reversible inhibition of transcription) to better follow the temporal regulation of these expression bursts. This would also be an ideal model to study the mechanisms controlling such rapid changes in histone acetylation and DNA methylation.

Minor points.

Page 3

The authors describe the effects of Tax on cell turnover. It would be fair to mention its powerful effect on plus strand transcription.

The authors state that, upon ex vivo culture of primary cells from infected patients, plus-strand expression is greatly increased while, the abundance of HBZ mRNA remains relatively stable. Although there is little doubt about the fact that plus strand expression is increased, previous studies showed that HBZ expression is increased as well (Rende et al., 2011).

Page 6

The CHIP and MeDIP analyses were carried out on different patients. It would have been more informative to compare these results in the same patients.

Page 10

The term CTCF mutant may be misleading as the mutation was introduced in the CTCF-binding site in the viral genome - not in the CTCF gene.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 30 Nov 2018

Charles Bangham, Imperial College London, London, UK

Minor points.

Page 3

The authors describe the effects of Tax on cell turnover. It would be fair to mention its powerful effect on plus strand transcription.

Response. A sentence mentioning the positive feedback by Tax on the 5' LTR promoter has been inserted in the 3rd paragraph in the Introduction section.

The author state that, upon ex vivo culture of primary cells from infected patients, plus-strand expression is greatly increased while, the abundance of HBZ mRNA remains relatively stable. Although there is little doubt about the fact that plus strand expression is increased, previous studies showed that HBZ expression is increased as well (Rende et al., 2011).

Response. We are currently investigating the quantity of *HBZ* mRNA in patient-derived PBMCs by smFISH. Therefore, in the present paper we confine the discussion on the HBZ expression in ex vivo culture.

Page 6

The CHIP and MeDIP analyses were carried out on different patients. It would have been more informative to compare these results in the same patients.

Response. Thank you for pointing out. We agree that it is informative to discuss results of different assays (ex. ChIP and MeDIP) in same patients, and also to compare the difference between patients for a same assay. To enable comparison of the results in this paper with data on the same subjects reported in other publications from our group, we have revealed in the revised paper the three-letter patient codes that are used in our other publications.

Page 10

The term CTCF mutant may be misleading as the mutation was introduced in the CTCF-binding site in the viral genome - not in the CTCF gene.

Response. We have systematically replaced the term "CTCF mutant" with "ΔCTCF-binding clone".

Competing Interests: No competing interests were disclosed.

Reviewer Report 31 August 2018

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Paul M Lieberman

Gene Expression & Regulation Program, The Wistar Institute, Philadelphia, PA, USA

HTLV-1 has complex transcriptional regulation in early and established long-term infections in T-cells. The Bangham group has previously shown that HTLV1 genomes establish an epigenetic patterns in latently infected T-cells, and how this corresponds to differences in viral transcription from the positive (Tax+) and negative (HBZ+) strands. Several intriguing features were previously observed, including a boundary of DNA methylation and a CTCF binding site at the pX locus. Additionally, a single cell study showed that transcription from the positive strand was dynamic, with some rare clonal bursts of TAX expression and viral reactivation, while the negative stand expression of HBZ was constitutive in all populations. In this new study, the authors further characterize the epigenetic control of HTLV-1 infected PBMCs and T-cell clones by comparing populations actively expressing Tax from those not expressing Tax.

The authors show by ChIP-Seq that histone modifications associated with transcription are activation at the 5' LTR and genome body of the Tax expressing population of HTLV-1 cells. In contrast, DNA methylation patterns as measured by MeDIP do not show significant variation in T-cell clones whether or not they express Tax. Sequence specific DNA methylation as measured by bisulfite sequencing shows some differences in Tax positive relative to Tax negative PBMCs from HTLV-1 carriers. Another major conclusion is that the CTCF binding to the proviral genome has no measurable function on viral gene expression or epigenetic patterning. Although this CTCF mutation is essentially negative data, it is important to report in context to previously observed findings and expectations of CTCF function in regulation viral transcription and epigenetics.

Specific Comments

1. The dynamic expression of the HTLV-1 positive strand needs some further characterization. What percentage of cells express Tax in the PBMC carriers and in the T-cell clones? Is this cell cycle, replicative age, or stress dependent?
2. The evidence that the population not expressing TAX is still expressing the negative strand is the single cell FISH. Can this also be detected by strand-specific RT-PCR to validate the FISH?
3. Fig. 3. ChIP-Seq data excludes the LTR. Understandably, since the LTR is duplicated, it may be problematic to assign specific signals to the 5' or 3' region. Nevertheless, the analysis of the combined LTR could be informative and should be included in the manuscript, either as a separate panel, or for a representative 5' or 3' LTR region for each histogram
4. Fig 3B does not provide a ChIP-Seq for CTCF on PBMCs. This is problematic since the paper focuses on the role of CTCF in various infection and latency scenarios. If CTCF does not bind in PBMCs, this should be reported, and the temporal or stochastic nature of CTCF binding should be further investigated. Is CTCF binding dependent on culture condition or clone specific?
5. Fig 9 is difficult to interpret without additional controls. A positive and negative control for CTCF binding, as well as an IgG control, should be included in each sample (Tax+ vs Tax-). Without these controls, or without the CTCF ChIP-Seq data in PBMCs from fig 3B, it is not possible to conclude that CTCF binds to the majority of genomes during primary infection in PBMCs. This raises the issue of whether CTCF binding to the HTLV1 genome is highly variable for different viral integrations and isolated clones. Along these lines, it may be worth examining multiple clonal isolates to determine how consistently CTCF binds to the proviral genome.
6. The title may be misleading since it is not clear that the 5' and 3' LTRs are analyzed completely and separately from each other.

Minor Issues:

1. A reference should be provided for statement on p3. 3rd paragraph of Introduction: "... transcription of... the plus and minus strand, respectively. (REF)"
2. p. 6, 1st column, Results section: "...In the 3' UTR and pX region, these three histone marks (H3K4me3, H3K9Ac and H3K27Ac) were constantly detected, regardless of Tax expression." I think this could be stated more clearly. Perhaps starting with a statement referring to the Tax negative population. Also, the 3'UTR is not examined, but rather the boundary adjacent to the 3' UTR is measured, according to the Figure Legend. Finally, the levels of H3K4me3 at the 3' end of the genome appears to be significantly enriched relative to the Tax positive population, perhaps suggesting these cells are expressing negative strand RNA and HBZ. This could be tested experimentally.
3. p6, 2nd column, 1st sentence: "...regardless of viral reactivation." I think "reactivation" may not be the appropriate designation, as TAX expression is the only readout, and this expression is dynamic through an unknown mechanism that may be distinct from viral reactivation that produces infectious particles. The status of HBZ expression should be tested or discussed.
4. It is surprising that there is so little DNA methylation detected in the LTR of the stable T-cell clones as measured by bisulfite sequencing (Fig. S2) compared to PBMCs from carriers (Fig. 5). Some consideration of this difference could be included in the Discussion section.
5. Fig. 5D. The bisulfite methylation may suggest that there is a trend toward selective methylation of primer region D in the Tax negative population. If the bisulfite conversion is not complete, this trend may suggest that DNA methylation is the mechanism of epigenetic silencing at the 5' UTR.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 30 Nov 2018

Charles Bangham, Imperial College London, London, UK

Specific Comments

1. The dynamic expression of the HTLV-1 positive strand needs some further characterization. What percentage of cells express Tax in the PBMC carriers and in the T-cell clones? Is this cell cycle, replicative age, or stress dependent?

Response. In HTLV-1-infected T cell clones, some 5-10% of the total population carry >4 plus-strand transcripts at any one time. We reported in our previous publication (Billman et al. Wellcome Open Research, 2017) that (1) HTLV-1-infected T cell clones have fewer *HBZ* mRNA molecules at the G0/G1 stage, and (2) a plus-strand burst (i.e. a rapid transcription) is more likely to be observed when a cell lacks detectable *HBZ* mRNAs. Indeed, the odds of a cell having a plus-strand burst at the G2/M stage is significantly lower than in cells in G0/G1 (Billman et al. 2017). These observations indicate an association between the plus-strand expression and the cell cycle in HTLV-1 T cell clones cultured in vitro.

In PBMCs isolated from HTLV-1 carriers, the HTLV-1 plus-strand reactivation is likely to be caused by environmental stress, as is observed in many other viruses. We are currently investigating the frequency and intensity of the HTLV-1 plus- and minus-strand expression during ex vivo culture of PBMCs by smFISH.

Paragraph 5 in the Introduction has been modified to include these important points, and the appropriate papers cited.

2. The evidence that the population not expressing TAX is still expressing the negative strand is the single cell FISH. Can this also be detected by strand-specific RT-PCR to validate the FISH?

Response. A possible alternative approach would be to sort the cells by flow cytometry for Tax protein and assay *HBZ* (minus strand) in the Tax-positive and Tax-negative fractions by RT-PCR. However, such approaches have certain limitations. Tax protein detection is less sensitive than smFISH, and crucially there is a time-lag between the onset of *tax* mRNA expression and protein expression; third, the PCR does not give information at the single-cell level. At present, we believe that smFISH is the most sensitive and specific technique available to detect mRNA, especially at the single-cell level.

3. Fig. 3. ChIP-Seq data excludes the LTR. Understandably, since the LTR is duplicated, it may be problematic to assign specific signals to the 5' or 3' region. Nevertheless, the analysis of the combined LTR could be informative and should be included in the manuscript, either as a separate panel, or for a representative 5' or 3' LTR region for each histogram

Response. We have revised Figure 3, Figure 7a and Supplementary Figure 1 to include reads aligned within the LTRs. We present those reads with grey colour in such a way that they can be differentiated from the rest of the reads that uniquely aligned.

4. Fig 3B does not provide a ChIP-Seq for CTCF on PBMCs. This is problematic since the paper focuses on the role of CTCF in various infection and latency scenarios. If CTCF does not bind in PBMCs, this should be reported, and the temporal or stochastic nature of CTCF binding should be further investigated. Is CTCF binding dependent on culture condition or clone specific?

Response. In Figure 3b, we omitted the ChIP-seq for CTCF in patient-derived PBMCs because of

the limited amount of materials. In the revised Figure 9 we show that HTLV-1 binds CTCF in PBMCs regardless of the plus-strand expression. Also, in our previous publication reporting the binding of CTCF in the HTLV-1 provirus (Satou et al. PNAS, 2016), we demonstrated that CTCF binds to various HTLV-1-infected cell lines as well as PBMCs isolated from HTLV-1-infected individuals.

It is documented that CTCF-binding is affected by methylation at one of the cytosine residue in the CTCF binding-site. In HTLV-1, CTCF binds to the pX region in the provirus where DNA is rarely methylated (Taniguchi et al 2005, op.cit.) (Figure 4). Therefore, we anticipate that CTCF binding to the HTLV-1 provirus shows little variation among culture conditions or provirus insertion sites. However, it should be noted that a lack of variation in CTCF-binding to HTLV-1 does not necessarily mean that the CTCF binding is strong. We discuss the CTCF occupancy as a surrogate measure of binding strength in the next point.

5. Fig 9 is difficult to interpret without additional controls. A positive and negative control for CTCF binding, as well as an IgG control, should be included in each sample (Tax+ vs Tax-). Without these controls, or without the CTCF ChIP-Seq data in PBMCs from fig 3B, it is not possible to conclude that CTCF binds to the majority of genomes during primary infection in PBMCs. This raises the issue of whether CTCF binding to the HTLV1 genome is highly variable for different viral integrations and isolated clones. Along these lines, it may be worth examining multiple clonal isolates to determine how consistently CTCF binds to the proviral genome.

Response. In the revised paper, we show the results (revised Figure 9) of new experiments in which we carried out CTCF ChIP assay on patient-derived PBMCs with two biological replicates, including a negative control (rabbit IgG) and the TC-1 locus as a positive control. The results show that CTCF binds to HTLV-1 after overnight culture, regardless of plus-strand expression. This observation is consistent with the conclusion that CTCF-binding has no discernible immediate impact on HTLV-1 expression.

The results in the revised Figure 9 also show that the CTCF ChIP signal at the HTLV-1 CTCF-binding site was lower than at the TC-1 locus (positive control for CTCF binding). CTCF occupancy (an approximation of binding strength) depends on the primary DNA sequence (Liu et al. Nature Biotechnology, 2015) and can be estimated by ChIP readout. We infer that CTCF occupancy at the HTLV-1 provirus is lower than that at TC-1.

6. The title may be misleading since it is not clear that the 5' and 3' LTRs are analyzed completely and separately from each other.

Response. We acknowledge that we have not explicitly identified the epigenetic modifications within the two LTRs. Also, as pointed out by both of the reviewers, an important finding is that CTCF appeared to have no discernible impact on the epigenetic modifications around the pX region or on spontaneous transcription. Therefore, we have altered the title of the revised paper: Spontaneous HTLV-1 transcription and epigenetic changes around the pX region are CTCF-independent.

Minor Issues:

1. A reference should be provided for statement on p3. 3rd paragraph of Introduction: "... transcription of... the plus and minus strand, respectively. (REF)"

Response. A reference (Kulkarni et al. 2018, review) has been inserted (Ref #5 in the revised paper).

2. p. 6, 1st column, Results section: "...In the 3' UTR and pX region, these three histone marks

(H3K4me3, H3K9Ac and H3K27Ac) were constantly detected, regardless of Tax expression.” I think this could be stated more clearly. Perhaps starting with a statement referring to the Tax negative population. Also, the 3'UTR is not examined, but rather the boundary adjacent to the 3' UTR is measured, according to the Figure Legend. Finally, the levels of H3K4me3 at the 3' end of the genome appears to be significantly enriched relative to the Tax positive population, perhaps suggesting these cells are expressing negative strand RNA and HBZ. This could be tested experimentally.

Response. Thank you for the suggestion. In the revised paper, we have defined the terms “5' LTR junction” and “3' LTR junction” at the beginning of the Results section, to clarify how we refer to the regions adjoining the 5' LTR and 3' LTR. Subsequently we systematically replaced “5' LTR” and “3' LTR” with “5' LTR junction” and “3' LTR junction” respectively.

In relation to this comment, two sentences have been altered in the revised paper: (1) In the last sentence in the first paragraph (Page 11) (“These results are inconsistent with the idea that CTCF imposes an epigenetic border in the HTLV-1 provirus and regulates the distinct modifications between the 5' and 3' LTRs.”), we replaced the phrase “between the 5' and 3' LTR” with “around the pX region”. (2) In the last sentence in the Discussion section, we replaced “in the 5' and 3' LTRs in HTLV-1” with “around the pX region in HTLV-1”.

Lastly, we state in the revised paper that H3K4me3 at the pX and adjoining the 3' LTR is higher in the Tax- population, presenting additional evidence obtained by ChIP-qPCR to make a quantitative comparison. The new data are presented in the revised Supplementary Figure 1 (Supplementary Figure 1a).

3. p6, 2nd column, 1st sentence: “...regardless of viral reactivation.” I think “reactivation” may not be the appropriate designation, as TAX expression is the only readout, and this expression is dynamic through an unknown mechanism that may be distinct from viral reactivation that produces infectious particles. The status of HBZ expression should be tested or discussed.

Response. We have preliminary evidence from smFISH analysis of HTLV-1⁺ PBMCs that cells that restore the plus-strand transcription also produce unspliced transcripts (*gag*), which is associated with production of HTLV-1 infectious particles. HBZ expression is currently under investigation with smFISH in conjunction with plus-strand transcripts at the single-cell level.

4. It is surprising that there is so little DNA methylation detected in the LTR of the stable T-cell clones as measured by bisulfite sequencing (Fig. S2) compared to PBMCs from carriers (Fig. 5). Some consideration of this difference could be included in the Discussion section.

Response. There are two major points to discuss concerning the difference between HTLV-1⁺ T cell clones and PBMCs. First, single T-cell clones are isogenic in terms of the genomic environment of the provirus insertion site, whereas the polyclonal population of PBMCs contains proviruses inserted in tens of thousands of unique sites, each with a distinct genomic environment. Second, it is plausible that a single cell with a particular pattern of DNA methylation was selected at the beginning of T cell cloning, and that pattern has been stably inherited in the clone. We infer that, at least in in vitro culture, DNA methylation at the 5' LTR promoter is stable, and has little impact on the spontaneous expression of the plus strand. We have added this point in the paragraph entitled “DNA hypomethylation in the plus-strand promoter is a prerequisite for viral reactivation” in the Results section.

5. Fig. 5D. The bisulfite methylation may suggest that there is a trend toward selective methylation of primer region D in the Tax negative population. If the bisulfite conversion is not complete, this trend may suggest that DNA methylation is the mechanism of epigenetic silencing at the 5' UTR.

Response. Our results show that the bisulfite conversion was highly efficient, as we could hardly see cytosine residues at non CpG sites after conversion. To estimate to what degree DNA methylation accounts for the provirus silencing, independently of the position effect, would require simultaneous identification of insertion site, DNA methylation and virus transcription in single cells. However, this is beyond the scope of the present paper.

Competing Interests: No competing interests were disclosed.
