Histone Acetylation and Methylation at Sites Initiating Divergent Polycistronic Transcription in Trypanosoma cruzi

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Patricia Respuela, Marcela Ferella, Alvaro Rada-Iglesias, and Lena Åslund

From the Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, SE-751 85 Uppsala, Sweden and the Program for Genomics and Bioinformatics, Department of Cell and Molecular Biology, Karolinska Institute, SE-171 77 Stockholm, Sweden

Trypanosomes are ancient eukaryotic parasites in which the protein-coding genes, organized in large polycistronic clusters on both strands, are transcribed from as yet unidentified promoters. In an effort to reveal transcriptional initiation sites, we examined the Trypanosoma cruzi genome for histone modification patterns shown to be linked to active genes in various organisms. Here, we show that acetylated and methylated histones were found to be enriched at strand switch regions of divergent gene arrays, not at convergent clusters or intra- and intergenic regions within clusters. The modified region showed a bimodal profile with two peaks centered over the 5’-regions of the gene pair flanking the strand switch region. This pattern, which demarcates polycistronic transcription units originating from bidirectional initiation sites, is likely to be common in kinetoplastid parasites as well as in other organisms with polycistronic transcription. In contrast, no acetylation was found at promoters of the highly expressed rRNA and spliced leader genes or satellite DNA or at tested retrotransposonal elements. These results reveal, for the first time, the presence of specific epigenetic marks in T. cruzi with potential implications for transcriptional regulation; they indicate that both histone modifications and bidirectional transcription are evolutionarily conserved.

The trypanosomatids, Trypanosoma cruzi, Trypanosoma brucei, and Leishmania major are human parasitic pathogens with a complex life cycle that comprises passage through different hosts and morphological states, requiring rapid and selective modulation of transcriptomes. Recent genomic sequenced have confirmed arrangement of trypanosome genes in large co-directional clusters (1), presumably expressed as polycistronic transcripts that are processed rapidly into individual mRNAs regulated mainly by post-transcriptional events (2). The transcriptional mechanisms in trypanosomes are poorly understood. Promoters of protein-coding genes, yet to be defined, are proposed to be located in the intergenic or strand switch region (SSR) between two divergent gene clusters arranged head-to-head, as shown previously in L. major (3, 4). The rarity of canonical transcription factors and regulatory DNA sequences points toward gene expression control through modifications of chromatin structures instead of the traditional modes of transcriptional control. Evidently, epigenetic regulation mediated through chromatin modifications would influence important processes such as antigen variation, virulence, and differentiation in trypanosomes and other protozoan parasites (5).

From bacteria to man, chromatin conformation often plays important roles in transcriptional control, DNA replication, repair, and recombination. Access to DNA is modulated by changes in the chromatin structure through methylation of DNA and post-translational modification (such as acetylation and methylation) of histones, specific histone variants, and chromatin-remodeling proteins. Trypanosome histones are unusually divergent in sequence, e.g. an atypical histone H1 lacking the globular domain (6), which contributes to the limited compaction of trypanosome chromatin with no further packaging into 30-nm fibers or metaphase chromosomes (7). However, altered chromatin condensation is observed at different developmental stages of T. cruzi; a higher compaction occurs in the non-replicative trypomastigote stage compared with the intracellular amastigote stage in mammalian hosts or with the epimastigotes stage in the insect vector. Variation in total mRNA levels is observed during development, with a reduction in trypomastigotes correlating with an increased amount of heterochromatin (8), suggesting a possible epigenetic contribution to transcription.

Apart from the canonical core histones, trypanosomes also harbor variants. Homologs to the histone H3 variants (H3.3, associated with active transcription, and CenH3, binding to...
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centromeres) have not been found in trypanosomes, which instead harbor a H3 variant found associated with telomeres (9). H2AZ, which have been linked to active transcription or prevention of the spread of heterochromatin in yeast, appears to have a different function in T. brucei, where its homolog does not co-localize to sites of transcription but dimerizes with a novel H2B variant (10). Chromatin-remodeling proteins relating to the SWI/SNF2 family have been found to regulate the synthesis of modified thymines in telomeric DNA in T. brucei, contributing to the epigenetic silencing of variant surface glycoproteins involved in antigen variation (11). Histone H3K79 methylation, mediated by DOT1 methyltransferase, is a modification associated with transcriptional activation and cell cycle control. In T. brucei, two DOT1 homologs were found to be important for mitotic cell cycle control (12). Histone acetyltransferase and deacetylases genes are present in the genome of trypanosomes. Four histone deacetylases (HDACs) have been studied in T. brucei, out of which two (DAC1 and DAC3) were shown to be essential for parasite survival and one crucial in cell cycle regulation (DAC4) (13). Also a SIR2-related protein showing both ADP-ribosylation and deacetylase activity, possibly involved in DNA repair, has been identified in T. brucei (14).

Histone H3 and H4 acetylation as well as histone H3K4 trimethylation are modifications associated with transcriptionally active genes and are typically found in the proximity of transcription start sites. The so-called “histone code” tends to be evolutionarily conserved; in particular, acetylation at H3K9/K14 and H4K5/K12 and trimethylation at H3K4 (H3K4me3) are linked to transcription rate (15). Of these, histone H4 acetylation and histone H3 trimethylation at Lys-4 have been found in trypanosomes (16–18). Because transcription initiation mechanisms, as well as the role of histone modifications, are largely unknown in trypanosomes, we were interested in mapping the in vivo location of acetylated histones H3/H4 and trimethylated histone H3K4 in different T. cruzi genomic loci implicated in transcription regulation. Here, we show a striking association of all three modifications to regions separating divergent gene clusters in the T. cruzi parasite, correlating with transcriptional initiation sites. This histone code profile shown here is likely to be the general pattern in kinetoplastid parasites and for polycistronic transcription in other organisms. Also, the acetylation status and nucleosome occupancy at promoters of the highly expressed rRNA and spliced leader (SL) RNA genes, as well as satellite DNA and some retrotransposons, were investigated.

EXPERIMENTAL PROCEDURES

Chromatin Immunoprecipitation (ChIP)—T. cruzi epimastigotes were grown as described (19). Approximately 10⁷ parasites were used per ChIP experiment following the protocol of Rada-Iglesias et al. (20) with some modifications. Parasites were washed twice with 1× PBS and resuspended in 25 ml of serum-free medium. Cross-linking was performed by adding formaldehyde to a final concentration of 1%. Precipitated DNA was resuspended in 50 μl of D2H0 for the ChIP reactions and 500 μl for the total inputs. All ChIP assays were done in three independent replicates. ChIP experiments were performed using α-acetylated histone H3K9/K14 (catalogue no. 06-599) and α-acetylated histone H4K5/K8/K12/K16 (catalogue no. 06-866) antibodies (Upstate) and α-histone H3 trimethylated Lys-4 (ab8580) and α-histone H3 (ab1791) antibodies (AbCam). Additional ChIPs with “no antibody” were used as controls. PCRs were performed using 0.5 μl of the resuspended DNA and corresponding primers (0.3 μM) (supplemental Table S1) in a total volume of 30 μl. PCR reactions were heated for 10 min at 95 °C and run for 15–32 cycles under the following conditions: 30 s at 95 °C, 1 min at 60 °C, 45 s at 72 °C, and a final step of 7 min at 72 °C. In the long range analysis, PCRs were carried out using a total of 17 different primer pairs spanning contig 7617 (supplemental Table S1). Two independent ChIP experiments were done for each antibody. The intensity of the bands was measured, and the mean value of two experiments was calculated. The ratio of positive to input values was normalized with respect to their mean, and log2 of the resulting values was used to build the graph (Fig. 3A).

Antibodies—ChIP assays were performed using antibodies raised against Tetrahymena acetylated histones H3 at Lys-9/ Lys-14, acetylated H4 at Lys-5/Lys-8/Lys-12/Lys-16, and trimethylated histone H3 at Lys-4, which were reported previously to recognize specifically the modified histones in the protozoan parasite Toxoplasma gondii (21, 22). All lysines are conserved in the T. cruzi histones H3 and H4 (supplemental Fig. S9). The three antibodies recognized only nuclear proteins in immunofluorescence assays of T. cruzi parasites (supplemental Fig. S9A). In Western blot (supplemental Fig. S9B), anti-acH3 antibodies recognized a band with an expected size corresponding to histone H3 (also recognized by antibody against total histone H3) as well as histone H4. Weaker bands of H2A/H2B were also detected. Anti-acetylated H4 antibodies bound preferentially histone H4 but also recognized the other histones. Anti-H3K4me3 antibodies recognized histones H3 and H2A/H2B (data not shown).

To further test the specificity of the antibodies, a peptide was designed corresponding to the 23 N-terminal amino acids of T. cruzi histone H4 containing the identified acetylated lysines (Lys-4, Lys-10, Lys-14) (17). This peptide was recognized by α-acH4 antibodies and competed for H4 binding in Western blot when antibodies were preincubated with the peptide (supplemental Fig. S10). Anti-acH3 antibodies did not recognize the acH4 peptide, and preincubation with the peptide did not affect the recognition pattern of α-acH3 antibodies in Western blots (supplemental Fig. S10). The same H4 peptide, including no acetylation, was not recognized by any of the antibodies (data not shown). Immunoprecipitation experiments to study the targets of the antibodies in native protein extracts of the parasite yielded insufficient amounts of protein to allow analysis by mass spectrometry. Total histone H3 protein was subjected to mass spectrometry, but characterization of N-terminal modifications was impeded because of difficulties in generating peptides of the appropriate size for analysis. Attempts were also made to enrich histone acetylation by treating parasites with the HDAC inhibitors trichostatin A (0.3 μM for 0, 6, and 24 h) and butyric acid (5 and 10 mM for 0, 6, and 24 h) with no success (data not shown).

Western Blot Analysis—Total protein extracts from T. cruzi epimastigotes were fractionated on NuPAGE 4–12% bis-Tris gel (Invitrogen) at 200 V for 35 min in MES running buffer.
MultiMark multicolored labeled (Invitrogen) was used as a protein size marker. The gel was transferred to a polyvinylidene fluoride membrane (GE Healthcare) at 80 V for 30 min on ice. The membrane was developed using an ECL Western blotting detection kit (see manufacturer’s instructions; GE Healthcare). Briefly, after 1 h of incubation in blocking solution, the membrane was washed and incubated for an additional hour with primary antibody. Primary antibody dilutions used in this protocol were as follows: α-acH3 and α-H3, 1:2000; α-acH4 and α-H3K4me3, 1:5000. Secondary antibody incubation was performed using rabbit IgG horseradish peroxidase-linked antibody (GE Healthcare) at 1:5000, and the result was detected on Hyperfilm ECL (GE Healthcare).

Immunostaining—T. cruzi epimastigotes were washed twice in 1X PBS and fixed in PBS containing 4% paraformaldehyde for 20 min. One drop of resuspended parasites was spotted onto each coverslips pretreated with poly-lysine (Sigma) for 30 min, and the cells were then permeabilized with PBS/0.3% Triton X-100. The slides were treated with Image-iT FX signal enhancer (Molecular Probes) for blocking background staining. An additional overnight blocking step was carried out in a filtered solution containing 2% (w/v) bovine serum albumin and 0.1% (v/v) Triton X-100 in 1X filtered PBS. All antibodies were used at a dilution of 1:200 in blocking solution for 1-2 h at room temperature. The coverslips were rinsed in 1X PBS and incubated with anti-rabbit Alexa 488 (Molecular Probes) with a dilution factor of 1:150 for 1 h. Finally the coverslips were rinsed with 1X PBS and mounted in ProLong Gold Antifade (Molecular Probes). For controls, parasites were also stained with secondary antibody alone at a dilution of 1:150. Images were acquired using a 510 Meta confocal microscope (Zeiss) equipped with a charge-coupled device camera. A ×63 oil immersion objective was used. Images were collected using LSM 5 Image Browser software and subsequently were prepared using Adobe Photoshop Elements 2.0.

Quantitative PCR (Q-PCR)—ChIPs were performed as described above, using α-acH3 and α-H3K4me3 antibodies. Q-PCR was run using the SYBR Green detection system in ABI PRISM 7900HT, typically using 0.5 μl of ChIP DNA/reaction. In parallel with the ChIP DNA, a serial dilution of the corresponding input DNA (1:2, 1:10, 1:50, 1:250) was also analyzed by Q-PCR. Ct values (where Ct is cycle threshold) were obtained in each case, and a standard curve for each primer pair was calculated based on the values of the input serial dilution. Based on those curves we inferred the amount of DNA (expressed in arbitrary units) in the ChIP DNA for each analyzed region. All genomic regions and primers employed are listed in supplemental Table S1. All Q-PCR reactions were performed in duplicate, unless the S.D. between signals was higher than 1, in which case a third replicate was obtained.

Cloning and Sequencing of ChIP Material—About 100 ng of the DNA precipitated with anti-acH3 antibody was amplified using the GenomePlex® Complete Whole Genome Amplification (WGA) Kit (Sigma) following the manufacturer’s instructions. The resulting DNA was purified using GFX™ PCR DNA and gel band purification columns (GE Healthcare) ligated into pGEM-T vector (Promega) and transformed into Epicurian Coli XL1-Blue (Stratagene). Amplified inserts or plasmid DNA from recombinant clones were sequenced by BigDye® Terminator version 3.1 (Applied Biosystems) and run on an ABI PRISM® 3700 DNA analyzer. Sequence analysis was done using Sequencer™ software.

RESULTS

Acetylated Histones H3/H4 and H3K4me3 Associate with Strand Switch Regions Separating Divergent Gene Clusters—The distribution of acetylated histones H3/H4 and H3K4me3 in 24 head-to-head (HH) and 24 tail-to-tail (TT) SSRs was analyzed in the T. cruzi CL Brener strain at the epimastigote stage using ChIP. Invariably, all analyzed HH SSRs were enriched with the modified histones (Fig. 1A and supplemental Fig. S5), whereas all TT SSRs except one (see below) showed low levels of histone modification. In amplified ChIP material, H3K4me3 gave the strongest signal, but the α-acH3 antibodies showed a more pronounced differential distribution compared with the α-acH4 antibodies (Fig. 1, A and C). The numerical data of the different enrichments of modified histones in HH versus TT SSRs, corrected for nucleosome occupancy (see below), are shown in supplemental Table S2 and Fig. 1C and as a box plot in supplemental Fig. S6. The estimated average values showed the highest enrichment level for H3K4me3 (10-fold), whereas acetylated H3 and H4 yielded an approximate 7- and 6-fold enrichment levels, respectively. Double bands were sometimes obtained as a result of sequence length polymorphism among homologous chromosomes because of the hybrid nature of CL Brener strain. Only one TT (contig 7271) showed some enrichment in acetylated and methylated histones (supplemental Fig. S5), which could be due to the presence of two tRNA genes carrying their own promoters. However, TTs 6288 and 7869 also contain tRNAs and yet showed neither acetylation nor methylation (supplemental Fig. S5 and Fig. 1A, respectively). The acetylation status of the analyzed regions did not differ among parasite strains, as similar results were obtained from α-acH3 ChIP experiments in other T. cruzi strains (data not shown).

We quantified the enrichment level of histone H3 acetylation by analyzing a subset of HH and TT SSRs using ChIP/Q-PCR (Fig. 1B). The enrichment varied among HH SSRs, but on average the values at these HH SSRs increased by a factor of 18 compared with those at the TT regions.

To confirm that the CHIP results were not the result of absence or inaccessibility of nucleosomes in TT regions, an α-H3 antibody recognizing modified as well as unmodified histone H3 was used to provide a measure of total nucleosome occupancy. Both the HH and TT regions were enriched for total histone H3, confirming the presence of nucleosomes (Fig. 1A and supplemental Fig. S5), although the enrichment of total histone H3 appeared to be higher at the HH regions. To analyze whether the difference in modified histones between HH and TT was simply due to variation in the amount of nucleosomes, the enrichment values for modified histones were corrected for nucleosome occupancy by dividing these with the enrichment levels of total histone H3. The resulting data showed that the modified histones indeed were overrepresented at the HH or divergent SSRs, as mentioned above (supplemental Table S2 and Fig. 1C). These experiments also verified that the αH3 and αH4 antibodies recognized the subpopulation of nucleosomes
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To examine whether intragenic acetylation had influenced our results, given that the HH and TT SSRs comprise the 5′- and 3′-ends of genes, respectively, we determined the acetylation pattern of a set of 5′- and 3′-UTRs positioned within the gene arrays at an average 20-kb distance from a HH SSR at different locations in the T. cruzi genome. No enrichment of acetylated histones H3 and H4 was observed at either the 5′- or 3′-UTRs (Fig. 2). We concluded that distribution of acetylation does not differ intragenically in T. cruzi and that acetylated histones were preferentially localized at HH SSRs. These results concur with the notion of the modification pattern expected for polycistronic transcription units. However, we cannot exclude the possibility that additional sites of transcriptional initiation do occur at locations other than HH SSRs. In T. brucei, RNA pol II has been co-localized in vivo with transcription factor TRF4 to the 3′-ends of a few genes associated with increased local transcription (25). Whether these sites are cryptic promoters is not known.

Acetylated Histones H3/H4 and H3K4me3 Are Present at Sites Initiating Bidirectional Transcription of Polycistronic Units—To determine the extent of acetylation and trimethylation surrounding a HH SSR and profile a larger region of the T. cruzi genome, we performed a long range ChiP analysis covering a genomic contig of 250 kb comprising one HH and one TT SSR (Fig. 3A). The genomic region was probed using the same set of antibodies and 17 primer pairs located both in intergenic regions and within predicted open reading frames. Again, the HH SSR was highly enriched in acetylated H3/H4 histones and H3K4me3, although loading of nucleosomes was equal along the whole region (Fig. 3A). Conservative estimates showed the acetylation to extend about 6 kb around the HH SSR encompassing the juxtaposed genes, supposedly embracing shared or separate initiation sites for bidirectional transcription on both strands. Two dips in the acetylation profile appearing at primer pairs 12 and 14 (Fig. 3A) are due to regions that are poorly amplified and not to a lower occupancy of nucleosomes. The trimethylated histone H3 profile showed some enrichment in the region preceding the HH SSR (Fig. 3A); however, the HH SSR

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with acetylated histones. The specificity of the antibodies used was tested as described under “Experimental Procedures” and shown on supplemental Figs. S9 and S10.

To identify genomic regions enriched in acetylated histones without any preselection, the immunoprecipitated α-acH3 DNA was amplified, cloned, and sequenced. Of in total nine sequences, seven were enriched and represented regions located in proximity to a HH SSR (50 bp to 6 kb). H3 acetylation at these sequences was confirmed by PCR analysis of ChiP material using specific primers (supplemental Fig. S11). Several of the cloned sequences were derived from parasite surface antigen genes, a reflection of the large number of SSRs located in subtelomeric regions, which harbor the main arsenal of such genes.

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FIGURE 2. No differential distribution of histone acetylation at 5' - or 3' -ends of genes. ChIPs using anti-acetylated histone H3 (α-acH3) antibody and a no antibody control (No ab.) were performed. PCR analysis of the resulting ChIP DNAs and corresponding input (total genomic DNA) is presented for a panel of selected 5'-UTRs (left) and 3'-UTRs (right). All UTRs are derived from genes located within gene clusters and on average at least 20 kb from a HH region.

of this contig is still about 14 times more enriched in H3K4me3 (supplemental Table S2).

Two HH SSRs were further analyzed at higher resolution using ChIP/Q-PCR to amplify ~150-bp-long fragments covering the HH region as well as parts of the flanking regions (Fig. 3B and supplemental Fig. S7). We observed two peaks of hyperacetylation as well as trimethylation downstream of each of the two ATGs separated by an unmodified region localized at the HH SSRs. Alternatively, the dip in the profiles could also imply a region depleted of nucleosomes, as described previously for yeast pol II promoters (26, 27), which are known to comprise transcription factor binding sites. Similarly, T. cruzi HH SSRs are rich in poly(dA-dT) stretches as seen for yeast promoters.

To verify that transcription initiates within a HH SSR of T. cruzi, nuclear run-on analysis was performed using strandspecific oligomers spanning the region (supplemental Fig. S8). Transcription of both the sense and antisense strands appeared to initiate within the unmodified part of HH SSR 7739. Transcription from the SSR was thus bidirectional, although the signal for sense strand transcripts was much weaker, as seen from the dot blots on the right segment of the SSR (Fig. S8A). Such difference in strength of the transcription signals between strands was also reported for the divergent SSRs on chromosome 1 in L. major (3). Transcription of both genes immediately flanking SSR 7739 was also verified by reverse transcription-PCR of steady-state RNA (supplemental Fig. S8B). Thus, our results correlate well with the findings from various organisms in which acetylated H3 and H3K4 trimethylation are found close to, but not within, the actual transcriptional start sites (15).

Acetylated Histones Are Not Associated with Promoters of Highly Expressed Genes—We investigated the acetylation status of previously known promoters in Trypanosoma spp., including the RNA polymerase I 18 S rRNA promoter (28) and the RNA pol II promoter for SL genes (29) encoding the 39-nucleotide-long capped SL spliced in trans to the 5'-ends of all mRNAs (30). We also tested the upstream sequences in T. cruzi that correspond to a region reported to initiate transcription of actin genes in T. brucei (31). No acetylated histone H3 or H4 was detected at promoters of the highly expressed 18 S rRNA and SL RNA genes (Fig. 4A). This could be because of a depletion of nucleosomes, as shown to occur at highly transcribed genes in other organisms (32). A similar status was found in T. cruzi, where ChIPs performed using α-H3 antibodies detected a very low amount of nucleosomes (Fig. 4A). Also, in Leishmania tarentolae, the SL promoter was recently shown to be devoid of nucleosomes (33). Furthermore, transcription of rRNA and SL RNA has several common features, including both promoter sequence and transcription factors, indicating a partly similar pattern of regulation (34) that might also include nucleosome depletion.

The two tandem repeat actin genes in T. cruzi are located next to a HH SSR, whereas in T. brucei these are located within a gene array in a head-to-tail arrangement. A transcription start site for the T. brucei actin gene was previously mapped around 4 kb upstream from the ATG (31). Comparative analysis of both the T. brucei and the T. cruzi 5' environment of actin genes revealed similarity in only ~200 bp located upstream of the T. brucei transcription start site and in a T. cruzi HH SSR (Fig. 4A, fragment a). Hyperacetylation spanned the entire T. cruzi HH SSR, amplified by three PCR primer sets, with the highest ratio (α-acH3/input) in fragment a, comprising the region of similarity (Fig. 4A). Whether the transcription start site region in T. brucei would be enriched in acetylated histones and provide an example of a unidirectional promoter remains to be investigated.

Several Repetitive DNAs Are Devoid of Acetylated Histones—To investigate the acetylation state of repetitive DNA, presumably part of the heterochromatin domains and hence transcriptionally inactive, ChIP assays as described above were performed on the T. cruzi satellite DNA, a 196-bp repeat constituting about 10% of the genome (35). As expected, no acetylated histones were detected in these repeats (Fig. 4B); however, there was enrichment of total histone H3, suggesting the presence of nucleosomes (data not shown). We also probed the T. cruzi retrotransposons SIRE and CZAR. The short, interspersed, repetitive element, SIRE, is highly abundant in subtelomeric regions and in the UTRs of several genes (36), whereas CZAR is a low copy number repeat interrupting a fraction of the SL gene repeat unit (37). Initial PCR analysis performed with SIRE internal primers on α-acH3 ChIP indicated hyperacetylation. However, only SIREs located within HH SSRs presented hyperacetylation when using primers discriminating between locations at HH SSRs or within gene clusters (Fig. 4B). Acetylation is hence not associated with SIRE elements per se, but there is an indirect association because of the location of these elements within HH SSRs. However, it is noteworthy that we found SIRE elements within or nearby 17 of the HH SSRs by in silico analysis, which would explain the initial enrichment. Also, no acetylation was found at CZAR retrotransposons; this result might also be a consequence of its genomic location within the SL gene repeat unit (37), which...
we showed above to be a region depleted of or with low amounts of nucleosomes.

**DISCUSSION**

The specific association of histone acetylation and trimethylation to HH SSRs comprising transcriptional initiation sites is likely to be a general pattern in trypanosomatids, as we have analyzed almost one-fifth of all HH (312) and TT (262) SSRs present in the annotated diploid *T. cruzi* sequence.5 A large number of SSRs are found in subtelomeric regions, where most of the genes encoding the various surface antigens in *T. cruzi* are located. Hence, several sequences from the cloned ChIP material enriched in acetylated H3 corresponded to surface antigen genes flanking the HH SSRs. The assayed histone modifications might thus also be regulating the expression of surface antigen genes, which are involved in parasitic evasion of the host immune system as well as cell targeting and invasion (38). Interestingly, histone acetylation is involved in selective expression of surface antigens and virulence genes in *Plasmodium falciparum* and *T. gondii* (21, 39). However, no histone acetylation has yet been shown to be associated with the active expression of variant surface glycoprotein genes in *T. brucei*, although its developmental silencing is chromatin-mediated (40).

No obvious sequence motif was found in common among all HH SSRs, except for regions rich in poly(A), GC, and polyT.

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5 D. Nilsson, personal communication.
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FIGURE 4. Chromatin state at RNA pol I and pol II promoters, satellite DNA, and retrotransposons. A, ChIPs using the indicated antibodies were performed together with a no antibody control. PCR analysis of enriched ChIP material and input (total genomic DNA) is presented for 18 S rRNA and SL promoters, as well as a possible transcriptional initiation site for the actin gene. Three primer pairs were designed to cover the 4-kb upstream region of the actin genes. B, PCR analysis of satellite DNA, CZAR, and SIRE retroelements in ChIP material. The SIRE retroelement was analyzed initially using internal primers and subsequently was reassayed using specific primers that flank the retroelement sequence and discriminate between locations in a HH region or within gene arrays.

(supplemental Fig. S12B). Most of the HH SSRs contained a small but distinct CpG island; however, GC-rich sequences were also present in TT SSRs. No common motif was found in the hypoacetylated fragments detected in ChIP/Q-PCR (Fig. 3A) except for the polypyrrimidine stretches. Transcription in both directions appears to be initiated within the short unmodified region according to our nuclear run-on results (supplemental Fig. S8), but whether these regions contain regulatory elements shared by the bidirectional transcription machinery will require further investigation. It is conceivable that HH SSRs form specific structures facilitating access to the transcriptional apparatus. The importance of the SSRs is reflected by the rarity of sequence polymorphisms within and around SSRs on chromosome I in T. brucei (41). Also centromere function has been assigned to a SSR on T. cruzi chromosome 3 (42). However, genome analysis shows that although gene clusters are syntenic between the trypanosomatid parasites, a surprisingly high number of breaks of synteny occur at or close to SSRs (1). It is noteworthy that histone H3 and H4 hyperacetylation, GC-rich sequences, and transcriptional activity are characteristics also shared by the origins of replication. HH SSRs could, in addition, be involved in replication initiation, allowing for the possibility of a coordinated regulation of transcription and replication (43).

The bimodular acetylation and trimethylation pattern revealed by higher resolution ChIP/QPCR analysis of HH SSRs (Fig. 3B and supplemental Fig. S7) is in perfect agreement with results from yeast and mammals, where in particular H3K4me3, H3K9ac, and H3K14ac modifications peak at the 5′-ends of transcribed genes (15). Our results are also supported by the location of acH3-enriched fragments within open reading frames close to HH SSRs (supplemental Fig. S11). The profile with broad peaks could, however, be further resolved using high-resolution probes or tiled arrays covering the entire region. Bidirectional promoters are also abundant in the human (44) and yeast (45) genomes. Such arrangements seem to influence mutual gene expression levels, as divergent gene pairs as well as close neighboring genes often are coexpressed, whereas genes in convergent directions (TT) have less transcriptional correlation (46). Mammalian bidirectional promoters are typically less than 1 kb in length and include a CpG island in 77% of the cases compared with 38% for unidirectional promoters (44). Both characteristics are also shared by the analyzed T. cruzi HH SSRs, with an average length of 773 bp (supplemental Fig. S12A), and CpG islands are present in 90% of the cases. Together, the histone modification patterns and nuclear run-on results strongly endorse the presence of initiation sites of bidirectional transcription in these regions, corroborating the transcriptional pattern found at such regions in L. major (3, 4).

We compared our results with human bidirectional promoters by mining the University of California Santa Cruz genome database, where data from the ENCODE consortium of acetylation profiles in human cell lines is available to the public. Histone H3 and H4 acetylation at human bidirectional promoters showed a pattern similar to that found in trypanosomes. In 13 examples (supplemental Table S3) for which expression data were available, both acetylated histone H3 and H4 peaks were found surrounding HH gene pairs as reported previously (24). This implies that divergent transcription from bidirectional promoters, as well as its regulation by the histone code, is an evolutionarily conserved process.

Clustering of genes is common among prokaryotes where functionally related genes, or operons, are co-transcribed into polycistronic pre-RNAs. Nematodes together with trypanosomatids are some of the few eukaryotes where polycistronic transcripts and trans-splicing have been characterized, e.g. in Caenorhabditis elegans about 15% of the genes are polycistronically transcribed (47). In trypanosomes, gene clusters comprise essential genes expressed at all stages of development without an apparent “operon” arrangement. It is therefore difficult to conceive a model in which the transcription of gene clusters is turned off and on, but a more or less constitutive transcription from the bidirectional “promoters” appears more realistic. Analysis of a 210-kb-long polycistronic transcription unit showed acetylated histones associated only to HH SSRs and not to any parts of the coding regions of the clustered genes or to intergenic regions (Fig. 3A). Such demarcation of transcription units by acetylation profile might be a general pattern in trypanosomatids and for polycistronic transcription units found in any other organism. The H3K4me3 profile also displayed a strong association to the HH region but showed, in addition, some enrichment at the end of the polycistronic unit including the TT SSR (Fig. 3A). Additional analysis is required to establish whether this enrichment reflects sites of transcriptional initiation (sense/antisense) or a specific chromatin structure. The nucleosome occupancy appeared to be more or less equal throughout the chromatin stretch regardless of intergenic or intragenic probing (Fig. 3A). A higher occupancy of nucleosomes appeared to be present at HH SSRs than at TT SSRs (Fig. 1A, supplemental Fig. S5 and Table S2). Whether this
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is because other modified or variant histones are present in the HH SSRs or there is a different chromatin structure in the TT regions affecting the accessibility of the antibodies is not known. In L. major, the transcription units have been suggested to terminate at tRNA genes located in TT SSRs (4). However, only three of the T. cruzi TT SSRs contained tRNA genes. TT SSRs could also contain nucleosome-free regions harboring elements or structures that facilitate dissociation of the colliding transcription machineries, similar to pausing sites for RNA pol II, which promote transcriptional termination in yeast and mammals (48). The T. cruzi TT SSRs vary in length (238–3680 bp), but some contain G-rich regions or sequences resembling such pausing sites.

Our findings show that transcription initiates in HH SSRs but do not exclude the presence of unidirectional or cryptic promoters within gene clusters. Random initiation and low levels of transcription of noncoding strands do occur in trypanosomes, suggesting a rather relaxed transcriptional regulation (3, 49). Whether sites reflecting these situations also would show association with histone modifications is not known. Our data are also supported by recent findings showing that acetylated histones H3/H4 and H3K4me3 are signatures of active promoters in the protozoan parasite T. gondii (21, 22).

An epigenetic contribution to transcription is indicated by changes in chromatin condensation observed at different developmental stages of T. cruzi (8). In yeast, the levels of several specific acetylation states found in the 5′-end of coding genes, such as H3K9ac and H3K14ac, correlate with transcription rate (27). As we found a substantial quantitative difference in acetylation as well as the trimethylation status at different HH SSRs (supplemental Table S2), there could be differences in transcriptional rates between T. cruzi gene clusters. Alternatively, this difference could result from lower amounts of nucleosomes in the amplified fragment. Comparing acetylation status at different developmental stages as well as measuring the initial transcription level would shed light on these questions. However, it is noteworthy that in T. cruzi as well as in L. major a substantial variation in the strength of transcription on the different strands was detected by nuclear run-on.

The association of acetylated histones H3/H4 and trimethylated histone H3 to sites of transcriptional initiation helps postulate a function for some of the recently detected post-translational modifications of Trypanosoma spp. Histones, where trimethylation of H3K4, acetylation of H2A (H2AK115/K119/K120/K125/K128) and H4 (H4K4/K10/K14/K57) have been verified (16–18). It was reported recently that in T. brucei acetylation of H4K4 is mediated by the histone acetylase HAT3 and is regulated in part by cell cycle (50). Also T. brucei histone variant H2Bv nucleosomes were recently shown to be enriched for H3K4 and H3K76 trimethylation, which could have an effect on transcription (51). Recent findings in which histones H3 and H4 were found to participate in RNA pol II and pol III complexes in L. major (52) further support our results. As histone H2A seems to be acetylated in trypanosomes and our antibodies showed some cross-reactivity, we cannot rule out contributions from this modified histone.

Investigations into epigenetic controls and the parasite enzymes involved therein are important for their potential as drug targets. Inhibitors of HDACs, such as trichostatin A, have shown antiparasitic activity against the apicomplexan protozoa (53). We attempted to enrich for acetylated histones by treating T. cruzi parasites with the HDAC inhibitors trichostatin A and butyric acid, but no changes in acetylation level could be detected by Western analysis (data not shown). This could be because of (i) low amounts of acetylated histones, (ii) a transient acetylation state of the histones, and/or (iii) poor effects of these drugs on T. cruzi HDACs under the experimental conditions used. Compared with other eukaryotes, trypanosomes would require a lower amount of acetylated histones, as hundreds of genes are transcribed from common initiation sites. As most of these promoters would be expected to be constitutively active, histone acetylation might therefore not be a transient state. However, other HDAC drugs have been shown to inhibit kinetoplastid parasite growth (54, 55), and it is noteworthy that some of these effects seem to differ between parasites and developmental stages. Whether these inhibitors influence the modification patterns shown here should be investigated.

In summary, our findings identify histone acetylation and trimethylation as indicators of regions for transcriptional initiation of divergent polycistronic transcription units in trypanosomes, and these regions contain bidirectional promoters. This arrangement appears to be a general set-up among trypanosomatid parasites, whereas known promoters for highly expressed genes have no acetylation and a very low occupancy of nucleosomes. The resemblance to mammalian systems suggests that the structure of bidirectional transcription, as well as the histone code, is evolutionarily well conserved.

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