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Expression of non-acetylatable lysines 10 and 14 of histone H4 impairs transcription and replication in *Trypanosoma cruzi*

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ABSTRACT

The histone H4 from Trypanosomatids diverged from other eukaryotes in the N-terminus, a region that undergoes post-translation modifications involved in the control of gene expression, DNA replication, and chromatin assembly. Nonetheless, the N-terminus of *Trypanosoma cruzi* histone H4 is mainly acetylated at lysine 4. The lysines 10 and 14 are also acetylated, although at less extent, increasing during the S-phase or after DNA damage, which suggests a regulatory function. Here, we investigated the roles of these acetylations by expressing non-acetylated forms of histone H4 in *T. cruzi*. We found that histone H4 containing arginines at positions 10 or 14, to prevent acetylation were transported to the nucleus and inserted into the chromatin. However, their presence, even at low levels, interfered with DNA replication and transcription, causing a significant growth arrest of the cells. The absence of acetylation also increased the amount of soluble endogenous histones H3 and H4 and affected the interaction with Asf1, a histone chaperone. Therefore, acetylation of lysines 10 and 14 of the histone H4 in trypanosomes could be required for chromatin assembly and/or remodeling required for transcription and replication.

1. Introduction

The dynamic acetylation and deacetylation of the N-terminus of the histone H4 is required for the assembly of H3/H4 dimers into the chromatin of several eukaryotic organisms [1,2]. These modifications act by regulating the binding of specific proteins that control the removal and addition of histones to the nascent DNA [3–5]. For example, in yeast, the acetylations of lysine 5 and 12 of histone H4 occur in the context of the H3/H4 dimer bound to the histone chaperone called anti-silencing function 1 (Asf1) through the action of the acetyltransferase complex Hat1/Hat2/Hif1 [6,7]. Once acetylated, the dimer can be transferred to the chromatin assembly factor 1 (CAF-1), or be directly used for chromatin assembly in the replication. Acetylation of H4K12 is also a characteristic of transcribing cells [8]. The acetylation of H4K16, for instances, modulates the chromatin organization in the interphase nucleus [9]. In *Drosophila*, it allows the binding of the chromatin-remodeling factor ISWI [10], affecting specifically some transcriptional events [11,12]. Therefore, acetylations in the histone H4 tail are important for the removal and reinsertion of histones from and to the DNA that is being replicated or transcribed [13].

The histones of Trypanosomatids are highly divergent compared to other organisms, mainly at the sites of post-translational modifications, in agreement to the existence of distinct regulatory mechanisms of transcription, replication and DNA repair [14–16]. However, some of these modifications appear to occur in conserved positions related to other eukaryotes. For example, the lysines 4, 10 and 14, are acetylated in the histone H4 of *Trypanosoma cruzi* and *Trypanosoma brucei* [17,18]. These lysines appear homologous to the K5, K12 and K16 of other organisms [14]. H4K4 is mostly acetylated (~70% of the total), decreasing after DNA damage, or in non-proliferative forms of *T. cruzi*. H4K10ac and H4K14ac are found at much lower levels (~1% of the total), increasing during S-phase or after DNA damage [19]. In *T. brucei,* these modifications do not appear to be reversible, occurring during the S phase of the cell cycle, possibly during chromatin assembly [20,21]. In addition, it has been shown by chromatin immunoprecipitation analysis that H4K4ac and H4K10ac are enriched in divergent regions of open

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reading frames of chromosomes, known to harbor transcription initiation sites for the polycistronic transcription [22,23], and also replication origins [24]. The reasons why the modifications are concentrated in these regions are unknown.

To understand the role of the lysine 10 and 14 acetylations in the *T. cruzi* histone H4, we generated parasites expressing non-acetylatable forms of the protein by replacing these lysines by arginines in an inducible manner. To follow the expression, sequences coding for the Myc-epitope were introduced in the C-terminus of the expressed histones. We found that these tagged histones H4 were appropriately expressed, imported to the nucleus and inserted into the chromatin. However, DNA replication was delayed and transcription was decreased. In addition, we observed an increased solubility of endogenous histones. As these modified histones were expressed at low levels and interacted anomalously with Asf1, we suggest that acetylations in the lysines 10 and 14 are required for chromatin assembly and/or disassembly in trypanosomes, although we cannot exclude a direct effect on the transcription initiation sites.

2. Material and methods

2.1. Parasites

Epimastigotes of *T. cruzi* (Y-strain) [25] were maintained in liver infusion tryptose (LIT) media supplemented with 25 μg/ml hemin, 10% fetal bovine serum at 28 °C [26]. The Y13i strain was obtained by transfection of the Y strain epimastigotes with 40 μg of the *Not*I linearized pLew13 plasmid [27] using the conditions described early [28]. The cells were selected in medium containing 0.5 mg/ml Geneticin G418 and cloned by limiting dilution. The number of parasites was determined by counting using a Neubauer chamber. Cell viability was determined by incubation of live parasites resuspended in saline phosphate buffer (PBS) at 5×10^6 parasites per ml with 10 μg/ml of propidium iodide for 10 min. After this incubation, the cells were analyzed by flow cytometry. Fluorescent cells were considered unviable. Positive controls were made with cells prefixed in 70% ethanol.

2.2. Cloning and gene expression

Parasites expressing exogenous histone H4 were prepared by PCR amplification of the histone H4 gene using the oligonucleotides shown in the Supplementary Table S1. Initially the wild type histone H4 gene was amplified using the genomic DNA of *T. cruzi* (Y-strain) with oligonucleotides TcH4Not e TcH4Nru. The PCR product was digested with *Not*I and *Nru*I and inserted into the pTCIndex-Myc plasmid [29] previously digested with NotI and *Eco*RV. The sequences with K10R and K14R substitutions were amplified by tandem PCR using the generated plasmid, which corresponded to the wild type gene, with the oligonucleotides TcH4K10mut or TcH4K14mut and MycTagRevBam. The products were reamplified with TcH4Not and MycTagRevBam oligonucleotides for cloning in the pTcIndex. The constructs were confirmed by sequencing and used to transfect the Y13i strain. The transfectants were selected in the presence of G418 and hygromycin B, both at 0.5 mg/ml. For expression induction, the cells were incubated with 2 μg/ml tetracycline added every 24 h.

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The recombinant histone H4 was obtained by PCR from the genomic DNA of *T. cruzi* (Y-strain) using the H4Nde and H4TcRev oligonucleotides and cloned in pET14b (Novagen). The protein was expressed in BL21De3 pLysS bacteria after induction with 1 mM isopropyl-thiogalactopyranoside and purified from inclusion bodies after solubilization in 6 M urea and affinity chromatography in Ni^{2+} Agarose (Qiagem). The obtained recombinant protein was dialyzed in PBS to remove urea and used to immunize mice. The recombinant histone H4 from *T. cruzi* with the Myc-tag at the C-terminus was obtained by expressing the wild type gene cloned into pTcIndexMyc after PCR using oligonucleotides H4Nde and MycTagRevBamH and insertion into the *Nde*I and *Bam*HI sites of pET26b plasmid (Novagen).

2.3. Western blot and immunofluorescence

The following primary antibodies were employed: Anti-Myc 9E10 [30], diluted 1:1000 from ascitic fluids; anti-H4 obtained from mice immunized with the recombinant *T. cruzi* histone H4 and used at 1:100 dilution; serum against the tri-acetylated synthetic H4 peptide, which reacted with non-acetylated and acetylated histone H4 and used at 1:100 dilution; affinity purified anti-H4K4, anti-H4K10, and anti-H4K14 antibodies, prepared and used as described [18]; and rabbit anti-histone H3 (Abcam) used at 1:1000 dilution, or prepared as described [31] and used at 1:20,000 dilution. For Western blotting, samples containing the equivalent of 0.2 to 5×10^6 parasites, or bacterial pellets were boiled in sample buffer and analyzed in 15% polyacrylamide gels containing 0.1% SDS (SDS-PAGE) using established procedures [32]. The proteins were transferred to nitrocellulose membranes for 20 min at 20 V using a semi-dry transfer system (BioRad) and stained with 0.5% Ponceau S in 1% acetic acid. Membranes were then treated with PBS containing 5% non-fat dry milk for 1 h and incubated with the antibodies diluted in 5% non-fat dry milk in PBS (blocking buffer) for one night at 4 °C. The blots were washed in PBS containing 0.1% Tween-20 and incubated with the secondary antibodies conjugated to peroxidase (Life Technologies) and chemiluminescent peroxidase substrate (ECL, Millipore). For quantitative analysis, bound antibodies were detected with the IRDye 680LT (anti-mouse IgG) or 800CW conjugates (anti-rabbit IgG) ((LI-COR Biosciences) using an Odyssey imaging system (LI-COR)).

For the immunofluorescence experiments, epimastigotes were washed and resuspended in PBS to 5×10^6/ml before adding to glass slides previously treated with 0.1% poly-L-lysine in water. After 20 min, attached cells were fixed with 4% p-formaldehyde in PBS, permeabilized for 5 min with 0.1% Triton X100, washed in PBS and blocked for 30 min with 1% BSA in PBS. After incubation with the indicated antibodies, the slides were washed, incubated again with the fluorescent conjugates (Alexa Fluor 488 and 594, Life Technologies) and mounted with ProLong Gold anti-fading (Life Technologies) in the presence of 10 μg/ml of 4′6′-diamidino-2-phenylindol (DAPI). Z-stacks were acquired using CellM software through an Olympus BX61 microscope equipped with a PlanApo (1.4 NA) coupled with an ORCA-R2 (Hamamatsu) digital camera. The stacks were processed for 3D deconvolution using AutoQuant X2.2 software (Media Cybernetics).

2.4. Cell cycle and transcription analysis

Parasites were collected in the mid-log-phase ($6–8 \times 10^6$ cells/ml), centrifuged at $2000 \times g$ for 10 min and adjusted to 1×10^7/ml before adding 1 mM 5-ethynyl uridine (EU) for the transcription assays. After 30 min at 28 °C, the parasites were washed three times with PBS, fixed and permeabilized in 50% ethanol for 20 min at 4 °C. These fixed and permeabilized cells were washed three times with PBS and the incorporated EU detected following click chemistry using Click-iT RNA Imaging Kit following the manufacture's instructions (Life Technologies). The cells were washed five times with PBS and analyzed by flow

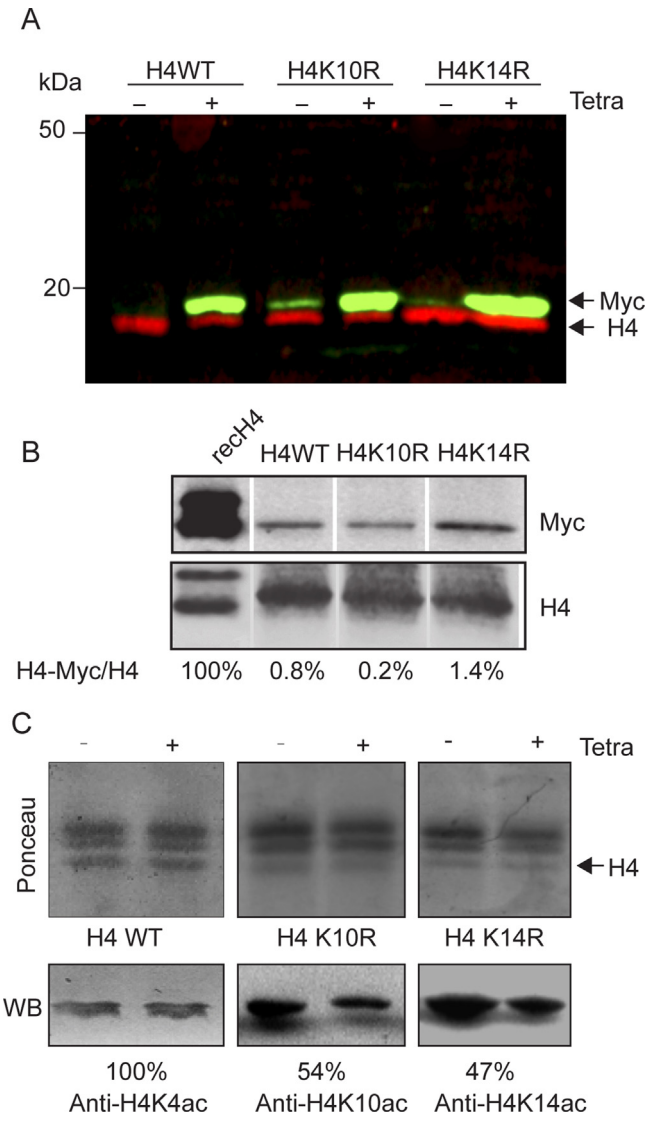

Fig. 1. Inducible expression of Myc-tagged histone H4. (A) Western blot of the extracts obtained from epimastigotes transformed with H4WT, H4K10R, or H4K14R (3×10^7 per lane) growth 4 days without (–) or with (+) 2 μg/ml tetracycline. The blot was incubated with anti-mouse IgG IRDye 680 for anti-Myc detection (green) and anti-rabbit IgG IRDye 800 conjugates for the anti-histone H4 detection (red). (B) Western blot (WB) containing an extract of *E. coli* expressing Myc-tagged histone H4 and extracts prepared from induced parasites. Both blots were probed with anti-mouse IRDye 680. The percentages at the bottom of the gels indicate the amount of exogenously expressed Myc-histone H4 relative to the total level of histone H4 (H4-Myc/H4), considering 100% as the reactivity towards the recombinant protein. (C) Western blot showing histones obtained from parasites transfected with H4WT, H4K10R and H4K14 after 4 days without (–) or with (+) 2 μg/ml tetracycline. The top panel shows the nitrocellulose membrane stained with Ponceau S and the bottom panel the labeling with affinity purified antibodies to acetylated H4K4, H4K10, and H4K14 revealed with anti-rabbit peroxidase followed by ECL (WB). The percentages at the bottom indicate the relative amount of each modification normalized by the loading calculated by the Ponceau staining.

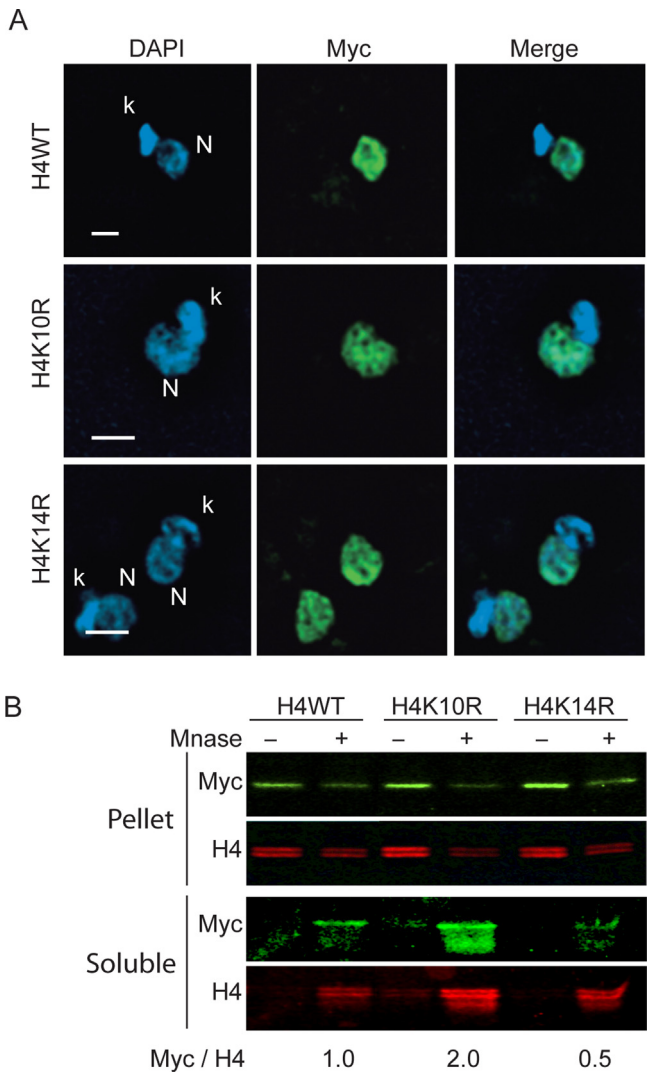

Fig. 2. Myc-tagged histone H4 mutants are incorporated into the nuclear chromatin. (A) The indicated *T. cruzi* cell lines were induced four days with 2 μg/ml tetracycline and labeled with anti-Myc antibodies (green) and DAPI, showing the nucleus (N) and the kinetoplast (k) in blue. Bar = 2 μm. (B) Western blots of the remaining chromatin pellets (Pellet) and supernatant (Soluble) of parasite extracts of the indicated cell lines that have been treated without (–) or with (+) Micrococcal nuclease (Mnase). The cells were all induced for expression of the tagged histones. In the top panels, the blot was probed with anti-Myc antibodies (green). In the bottom panels with unpurified anti-histone H4 antibodies from rabbits (red). The numbers below panel B indicate the relative solubilization (Myc/H4) compared to H4WT.

cytometry using the FL1 detector of the BD Biosciences Accuri C6 cytometer.

For cell cycle analysis, exponentially growing cultures (~1×10^7/ml) were incubated in LIT medium with 100 μM de 5-ethynyl-2′-deoxyuridine (EdU) for 2 h at 28 °C. The parasites were then collected by centrifugation (2000 × *g*), washed twice with PBS and resuspended in 500 μl of cold 50% ethanol. After 20 min on ice, the parasites were centrifuged, washed in PBS, and treated with 20 μg/ml RNase for 30 min at 37 °C. Parasites were centrifuged once more, washed and resuspended in the labeling buffer, and processed by using the Click-it® EdU Flow Cytometry Assay Kit (Life Technologies) according to the manufacturer instructions. The parasites were washed 3 times with PBS and incubated with the Cell Cycle 633 (Life Technologies) for 30 min. After a new round of washes, the cells were analyzed by using the Accuri II C6 flow cytometer.

2.5. Asf1 pull down assays

The recombinants of nuclear Asf1 (Asf1n) and cytoplasmic Asf1 (Asf1c) from *T. brucei* fused to the C-terminus of the maltose binding protein (MBP), prepared as described previously [31], were expressed in *Escherichia coli* BL21 DE3 after induction for 3 h at 37 °C with 1 mM isopropyl β-D-1-thiogalactopyranoside. The recombinant proteins were obtained from the soluble fraction of bacteria lysates prepared in 40 mM Tris–HCl pH 7.4, 0.4 M NaCl, 2 mM EDTA and 20 mM-β-mercaptoethanol by passage through a French Press.

The soluble material was incubated with Dextrin Sepharose High Performance (GE Healthcare Life Sciences) equilibrated in the same buffer. After 15 min at 4 °C, the resin was washed with 10 volumes of 20 mM Tris–HCl pH 7.4, 0.2 M NaCl, 1 mM EDTA and 10 mM β-mercaptoethanol (wash buffer). Bound Asf1s were immediately used for the pull down assays. Epimastigotes from the different lines were harvested by centrifugation at 2000 × *g* for 5 min at 4 °C and washed once in cold PBS. Parasite pellets were then suspended in lyses buffer [25 mM HEPES, pH 7.5, 150 mM KCl, 10% glycerol, 12.5 mM $MgCl_2$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride and a cocktail of EDTA-free protease inhibitors (Roche)] and lyzed by three freeze and thaw cycles, followed by 20 sonication rounds (30 s on and 15 s off, 30% output) using a Branson Sonifier digital D-250 (Emerson Industrial Automation). Cell extracts were cleared by centrifugation (10.000 × *g*, for 10 min 4 °C) and the supernatants were treated with 10 μg/ml DNaseI (Thermo Scientific) and 10 μg/ml of benzonase nuclease (Sigma) for 1 h at 37 °C. The obtained extracts, corresponding to ~1×10^8 parasites, were then incubated with the resin containing the adsorbed MBP fused to Asf1s at 4 °C with shaking. After 16 h, the mixtures were collected by centrifugation, washed with 10 volumes of wash buffer. The final pull down samples were incubated with SDS-PAGE sample, boiled, and submitted to SDS-15% PAGE and western blot as described above.

3. Results

3.1. *Myc-tagged histone H4 carrying mutations in the acetylation sites were expressed in an inducible manner and affected the levels of H4K10 and H4K14 acetylation in* T. cruzi

To modify the levels of specific histone H4 acetylation we generated parasites expressing histone H4 tagged with the Myc epitope at the C-terminal using a vector that integrates in the genome by homologous recombination into the rDNA locus of *T. cruzi*. This vector (pTCIndexMyc) allows expression after addition of tetracycline in cells containing the T7 polymerase and the tetracycline repressor [29]. Epimastigotes of the strain Y13i were transfected with the pTcIndexMyc vector carrying the wild type or the mutated forms of the histone H4. After hygromycin selection, the resulting parasites expressed the proteins in the presence of tetracycline, as revealed by the anti-Myc antibody (Fig. 1A). Myc-tagged proteins migrated slower in the gel due to the presence of the extra-C-terminal region. To quantify the amount of Myc-tagged histone H4 expressed in each cell line, we compared the signals obtained in Western blots using the anti-histone H4 and anti-Myc relative to the signals in the recombinant Myc-tagged histone H4 obtained in *E. coli*. As shown in Fig. 1B, the signal with anti-Myc was much higher in the *E. coli* recombinant compared to the signal obtained in parasites (top panel) while the signal with anti-H4 antibodies was similar. The recombinant H4 appeared as a doublet. It could be a gel artifact or the protein underwent proteolysis in bacteria. Anyway, the strong reactivity of recombinant containing the Myc with the Myc antibody, compared to the anti-H4, indicated that a small quantity of the total histone in the parasite contained the tag. The numbers below the panel represent ratios of total histone H4 containing the Myc-tag and total histone H4. This was estimated by infrared imaging considering the signals obtained with the anti-Myc antibodies when using similar amounts of total histones. Therefore, the exogenous histone corresponded to about 1% of the total amount of endogenous histone of the cells. For this reason, we were unable to detect the exogenous histone with anti-H4 antibodies. Interestingly, the expression of these low levels of the mutated, but not the wild type H4 decreased the amounts of H4K10 and H4K14 acetylation, without affecting the quantity of total histones, as detected by Ponceau staining (Fig. 1C). These results suggest that the expression of non-acetylatable K10 and K14 forms of histone H4, even in small amounts, was sufficient to interfere in the total levels acetylation at these positions, already corresponding to a small percentage of total histone H4 of *T. cruzi* [18].

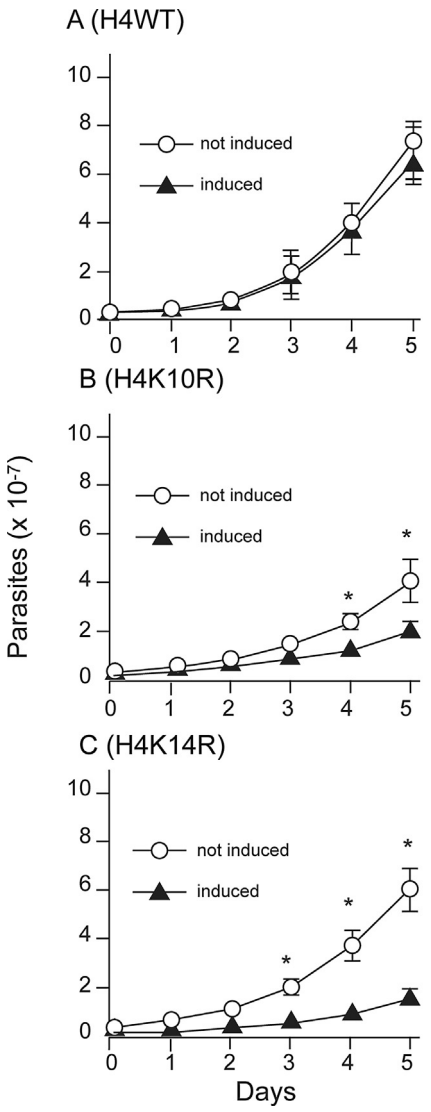

Fig. 3. Parasites carrying H4K10R and H4K14R have a decreased proliferation rate. Epimastigotes carrying the tagged wild type histone H4 (WT) (A), the H4K10R (B), or the H4K14R (C) were cultured in the absence (open circles) or presence (black triangles) of 2 μg/ml tetracycline. Values represent the number of parasites per ml (mean ± standard deviation) of biological triplicates. The differences indicated by (*) have *p* values <0.05, as determined by Student's *t* test.

3.2. *Mutated histone H4 are incorporated into the chromatin*

Immunofluorescence analysis revealed that wild type and mutated versions of the Myc-tagged histones were incorporated into the parasite nucleus (Fig. 2A). No Myc labeling was found in the kinetoplast or other cellular organelle, indicating that the Myc at the C-terminus of histone H4 did not affect nuclear import and that acetylation of lysine 10 and 14 were not required for the nuclear delivery of histone H4. Myc labeling was not detected in non-transfected cells (not shown). Interestingly, the nuclear labeling of the non-acetylated histones (H4K10R and H4K14R), as observed with anti-Myc antibody, differed from nuclear regions occupied by acetylated histones, as detected with specific antibodies for the

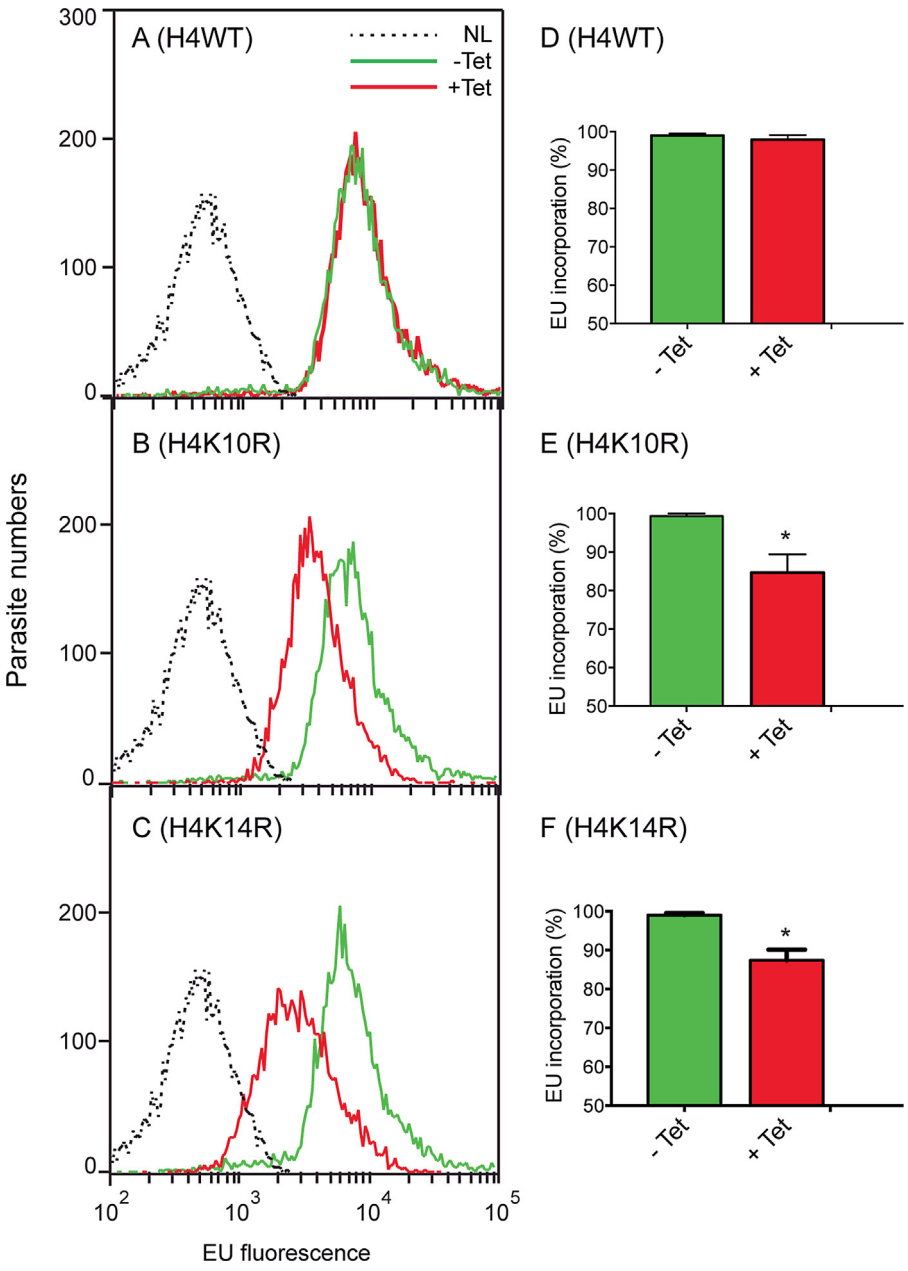

Fig. 4. Parasites expressing the mutated histone H4 have a decreased transcriptional activity. Epimastigotes containing the H4WT (A), the H4K10R (B) or H4K14R (C) genes were maintained at the exponential growth phase in the absence (−Tet) or presence (+Tet) of tetracycline for 4 days. The cells were then washed and incubated at same densities with with EU for 30 min, submitted to the Click reaction with the Alexa 488 probe and analyzed by flow cytometry. The figures show the histograms of unlabeled (NL) parasites for comparison. The experiments represent an example of biological triplicates. Panels (D) to (F) show the relative incorporation of EU in the triplicates. *Indicates a significant difference calculated by *t*-test ($p < 0.05$). (For interpretation of the references to colour in this figure legend reader is referred to the web version of this article.)

K10ac and K14ac (Fig. S1), suggesting that histones with acetylated lysine 10 and 14 not always occupy the same nuclear loci, as seen earlier [19].

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2015.11.001.

To further demonstrate that Myc-tagged histones formed nucleosomes, extracts containing intact nuclei from parasites expressing exogenous wild-type histone H4 (H4WT) and the mutated H4 versions (H4K10R and H4K14R), were treated with Micrococcal nuclease, and used for western blot with anti-histone H4 and anti-Myc antibodies (Fig. 2B). As Micrococcal nuclease is known to release and solubilize nucleosomes, the fact that part of the Myc-labeled histones became soluble, as found for the histones in general, confirmed that the presence of Myc, or the absence of acetylation at lysine 10 and 14 on histone H4 did not impaired their incorporation into nucleosomes. Interestingly, the solubilization after Micrococcal nuclease was higher in the H4K10R and lower in H4K14R compared to the H4WT.

3.3. Expression of mutated H4K10R and H4K14R affects cell growth

The presence of wild type histone H4 containing the Myc-tagged did not affect *T. cruzi* proliferation (Fig. 3A). In contrast, parasites expressing H4K10R and H4K14R showed a decreased proliferation, as compared to non-induced cell lines (Fig. 3B and C). A reduced proliferation was also observed for non-

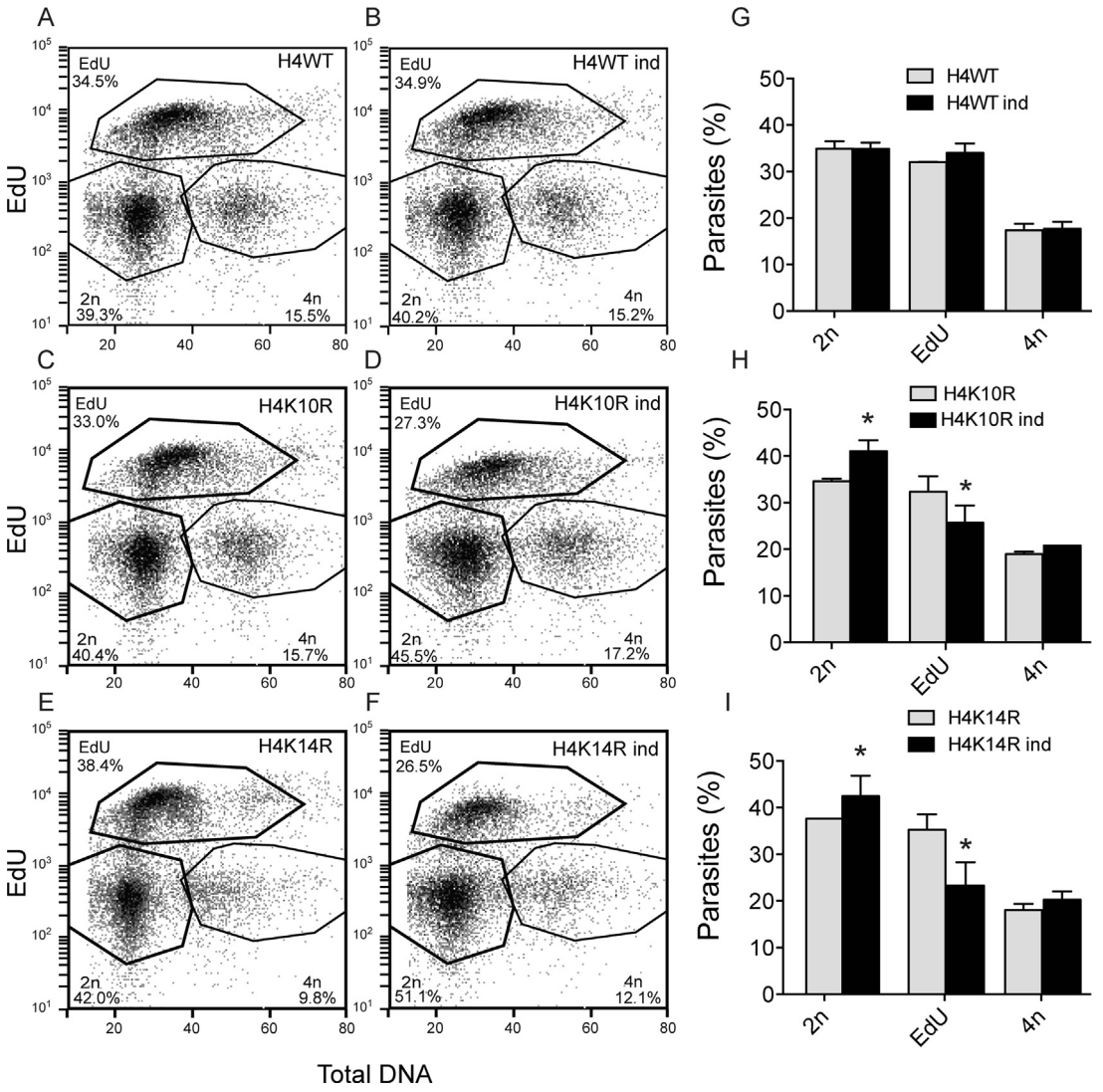

Fig. 5. H4K10R and H4K14R mutants have a decreased number of cells in S phase. Non-induced (A, C and E), or induced (B, D and F) parasites of the indicated lines were incubated with EdU for 2 h and then processed for the Click reaction with Alexa 488 and for the total DNA labeling with the Cell Cycle 633, as described in Methods. The panels show the EdU incorporation versus total DNA labeling. The numbers are the percentage of EdU labeled cells (cells in S), cells with 2n (cells in G1) and 4n (cells in G2) in the total population. Panels (G) to (I) show the mean percentage and standard deviations of each labeling in triplicate experiments. *Indicates a significant difference calculated by *t*-test ($p < 0.05$).

induced mutated cell lines, probably because the expression of the exogenous histones was leaky (see Fig. 1A). Importantly, the expression of the mutated H4 did not cause cell death. The parasites remained motile and viable, and showed no incorporation of propidium iodide. In addition, removal of tetracycline restored the growth rate to the non-induced conditions after 48 h, with identical growth compared to the non-induced cultures.

Expression of mutated H4K10 and H4K14 decreases transcription and caused replication arrest Next, we examined the transcription rates of the different cell lines after induction by tetracycline. We labeled the parasites with EU, a cell permeable ribonucleotide analog and measured its incorporation by the Click reagent containing Alexa 488. Non-induced, or cells induced to express the wild type histone H4 gene were equally labeled after 30 min of incubation with EU (Fig. 4A). In contrast, a decrease in EU incorporation was observed when cells were induced for H4K10R and H4K14R expression (Fig. 4B and C, respectively). A quantitative analysis indicated a 30% reduced transcription rate in these cells (Fig. 4D–F).

The parasite lines carrying the wild type and mutated histone H4 genes were also tested for the incorporation of EdU, a deoxyribonucleotide analog, which is incorporated during DNA replication. As shown in Fig. 5, parasites expressing H4K10R and HK14R presented a diminished amount of cells in S phase when compared to non-induced parasites or parasites expressing the wild type histone H4. In addition, we observed an increased number of parasites in G1 and less in G2, indicating a delay during the S-phase, or between G1 and S phase in three independent experiments. Although non-transfected cells showed a larger incorporation of EdU compared to parasites expressing H4WT, it presented a similar distribution in the G1 and G2 populations (Fig. S2), indicating that expression of the Myc-tagged histones affected minimally the cell cycle.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.molbiopara.2015.11.001.

3.4. Expression of H4K10R and H4K14R induces endogenous histone H3 and H4 solubilization

To gain some insight why the lack of acetylation of H4K10 and H4K14 affected transcription and replication, we compared the

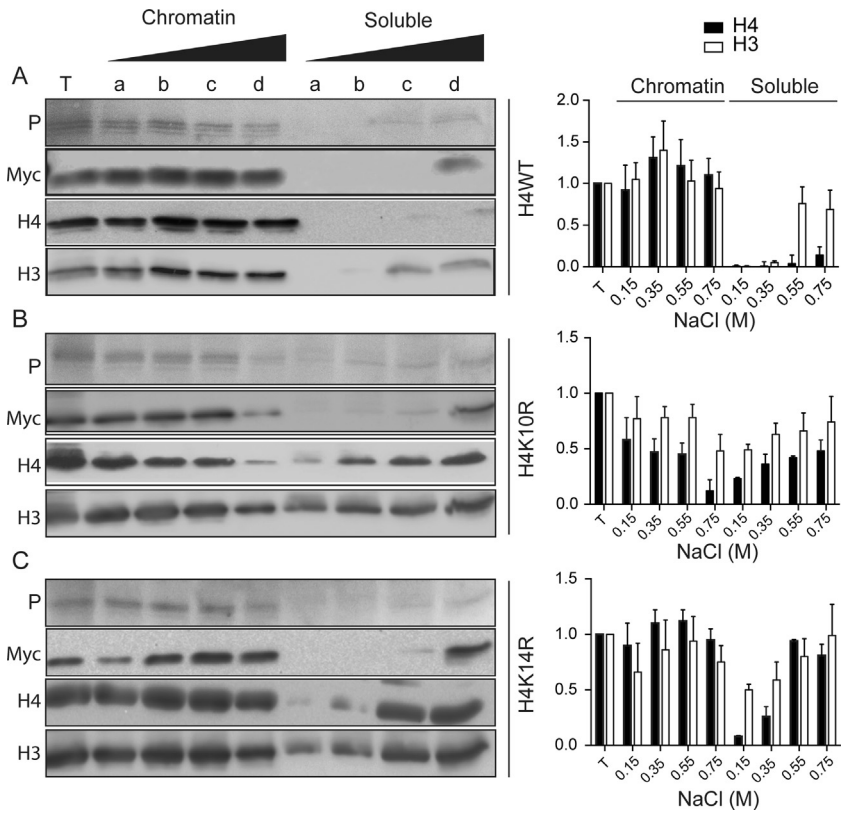

Fig. 6. H4K10R and H4K14R increased the amount of soluble histone H3 and H4. Exponentially growing parasites induced for the expression of H4WT (A), H4K10R (B) or H4K14R (C) were collected by centrifugation (2000 g, 10 min), washed in PBS, divided in five different microcentrifuge tubes and further centrifuged. One tube was directly lysed in 1X SDS-PAGE sample buffer (T). The other four tubes were lysed in PBS containing 1% Triton-X100 in the presence of protease inhibitors (cComplete, EDTA free, Roche) and the following final concentrations of NaCl: 0.150 M, 0.350 M, 0.55 M, 0.75 M, respectively samples a to d. After 20 min on ice, the tubes were centrifuged at 4 °C (10,000 × *g*, 20 min). The resulting precipitates were resuspended with the original volume of PBS, and an aliquot of the resuspended precipitate and the supernatant were mixed with one volume of 2X SDS-PAGE sample buffer. The top panels show the nitrocellulose membranes stained Ponceau S, and the bottom ones, blots revealed with anti-Myc, anti-H4 and anti-H3 antibodies followed by detection using ECL. Similar results were obtained in three independent experiments. The graphics on the right side represent the means and standard deviations of these experiments.

extractability of histones from chromatin by using increasing concentrations of NaCl. The solubilization of the tagged protein and the endogenous histone H3 and H4 were only detected at higher salt concentrations (>0.75 M NaCl) in cells transfected with the wild type Myc-tagged histone H4 (Fig. 6A). In contrast, large amounts of histone H3 and H4, but very few Myc-tagged histone H4, were extracted at lower salt concentrations from H4K10R and H4K14R expressing parasites (Fig. 6B and C). Remarkably, histone H3 was extracted at concentrations as low as 0.15 M NaCl (lane a). This result indicated that the presence of non-acetylatable histone H4 at lysine 10 and 14 increased the solubility of endogenous histones H3 and H4, while the non-acetylatable histone remained associated with the chromatin.

3.5. *Modified histones bind differently to the histone chaperone Asf1*

As the mutated histones were expressed at low levels and were able to induce large changes in the histone solubility, we hypothesized that the lack of H4 acetylation could disturb the assembly and disassembly machinery, because it is present at much lower levels when compared to the histones. Therefore, we tested the binding of the H3/H4 dimer to Asf1, a key histone chaperone. The H3/H4 dimers were obtained from parasites expressing H4WT, H4K10R and H4K14R. Parasites were lyzed and the chromatin solubilized by sonication. Histones complexes were released from the nucleosomes by extensive nuclease digestion and incubated with the cytosolic and nuclear Asf1 from *T. brucei* (Asf1c and Asf1n) fused to the maltose binding protein, as described [31]. Bound histone H3/H4 dimers were collected with dextrin-coupled beads. In all cases, Myc-H4 could be solubilized from the chromatin after nuclease digestion, further confirming its incorporation into nucleosomes (Fig. 7A). In addition, all mutants were collected with the Asf1 beads. Interestingly, the interaction with nuclear and cytosolic Asf1 was reduced in the case of H4K10R and increased in H4K14R, relative to H4WT, considering the amount of bound H3, suggesting that the modifications were affecting the interaction with the histone chaperone.

4. Discussion

In several organisms, addition and removal of histones from the chromatin during the processes of transcription, replication and DNA repair depends on histone H4 tail acetylation [12,33–36]. Here we showed that expression in *T. cruzi* of histone H4 containing a non-acetylatable amino acid in the positions 10 and 14 had an effect on transcription, replication, and consequently, parasite multiplication. The absence of acetylation did not prevent nuclear import and histone H4 incorporation into the chromatin, resulting in a decreased amount of total acetylated K10 and K14. Furthermore, the expression of low levels (≅1% of the total) of non-acetylatable H4 caused an increase in soluble H3 and H4. As the non-acetylatable histones showed different binding to Asf1, we proposed that the reduction in the amount of endogenous acetylated K10 and K14, the reduced transcription, replication, was due to the prevention of an adequate flow of histones during chromatin assembly, remod-

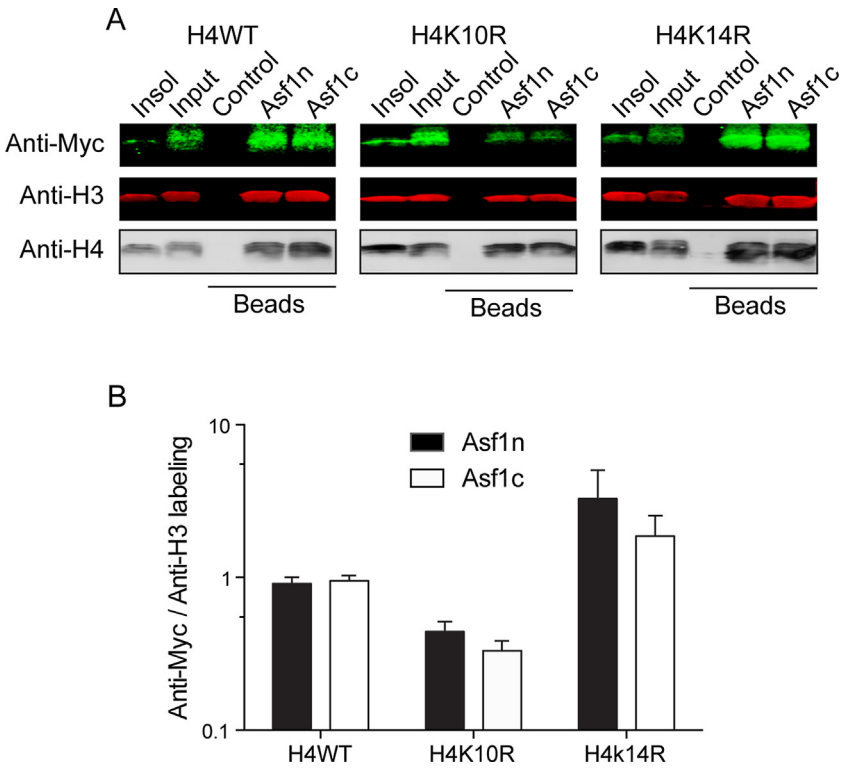

Fig. 7. Asf1 interaction with H3/H4 dimers is affected by the presence of mutations in the histone H4. (A) Epimastigotes were induced with 2 μg/ml tetracycline during four days and whole extracts of parasites (5×10^8 cells) were prepared by freeze-thaw followed by sonication and treatment with nucleases as described in Methods. An aliquot of the insoluble fraction (Insol) and of the total solubilized fraction (Input) was removed and loaded in the SDS-PAGE. The remaining soluble fractions were incubated at 4 °C with the amylose resin coupled to the recombinant Asf1c and Asf1n, or with the resin pretreated with an unrelated bacterial extract (control). The materials bound to resin were incubated with sample buffer and 50% loaded in the gel. Western blots were performed with the anti-Myc and anti-H3 and revealed by using IRDye secondary antibodies. The membranes were stripped and incubated with anti-H4 and reveled by ECL. (B) Quantification showing the signals obtained with the anti-Myc relative to the anti-H3 quantified using the Odyssey reader. The numbers are mean relations of two independent experiments. (For interpretation of the references to colour in this figure legend reader is referred to the web version of this article.)

eling or disassembly. It is unlike that the expression of H4K10R and H4K14R caused these effects by competing and displacing the endogenous histones from the chromosomes, as observed when overexpressing histones in eukaryotic cells [37], but we cannot exclude the possibility that transcription and replication was also affected by changes in the initiation regions of the chromosomes.

Our data allowed us to exclude the possibility that the Myc-tagging in histone H4 acted on the growth and replication, as cells containing the tagged wild type histone were not disturbed. Moreover, both the wild type and the mutated Myc-tagged histones H4 were extractable in high salt solutions, as other histones, becoming soluble after addition of Micrococcal nuclease, which solubilizes nucleosomes. We cannot, however, eliminate the possibility that the presence of the Myc sequence could eventually affect the nucleosome packing, as the C-terminus of histone H4 is located between the two tetramer forming each nucleosome [38].

We observed a decreased incorporation of EU, suggesting a decreased transcriptional rate when histone H4 contained mutated K10 or K14. The primary effect of expressing non-acetylated histones is not through a direct replacement of the modified histones in transcription sites, as they are not necessarily incorporated or modified in a particular region, differently to what happens for acetylated H4 [39].

The fact that large amounts of histone H3 and H4 were extractable in the parasites expressing H4K10R and H4K14R when using low salt concentrations, could explain the reduced transcription and replication. The presence of more soluble histones could indicate a replication and transcription arrest due either to the absence of nucleotides, a DNA damage response, or absence of enough histone chaperones and chromatin remodeling proteins [40]. As soluble or non-chromatin associated histones are usually bound to Asf1 [7], our results suggest that non-acetylatable histone H4 at positions 10 and 14 could prevent histones recycling through the chaperones. The fact that exogenously expressed H4 remained associated with the chromatin when the endogenous histones H3 and H4 were extractable further implies that acetylation could be required for removing the mutated histones, and/or binding to Asf1.

The decrease in transcription and replication can also be a consequence of changes in the putative sites of transcription initiation, which are located in regions of the chromosomes enriched in H4Ac [39,41]. To verify changes in the transcriptions sites, we probed these regions for accessibility to restriction enzymes as described [42], and we did not find differences (data not shown). Chromatin immunoprecipitation analysis would be necessary to elucidate whether transcription sites were specifically affected.

We also found that the cell cycle of cells expressing H4K10R or H4K14R was also altered. The mutants had a decreased S-phase progression resulting in more cells in G1, in agreement with a decreased replication rate. As in the case of transcription reduction, replication origins could be disturbed, as it starts on sites enriched in post-translationally modified histones [24]. Another possibility that cannot be excluded is that expression of the mutated histones induced a stress response, decreasing replication initiation.

Recently, we have shown that *T. brucei* contains two distinct Asf1, one located in the cytosol that shuffles into the nucleus in S phase and the other found exclusively in the nucleoplasm throughout the cell cycle [31]. The nuclear Asf1 was shown to require post-translational modifications to interact with the H3/H4 dimer. Our results showing the changes in the binding of H4K10R and H4K14R to Asf1 suggests that chromatin remodeling was, in fact,

affected by the expression of mutated H4. The impairment of acetylation in the K10, and K14 affected the interaction and block Asf1 function during chromatin remodeling, also observed when Asf1 and other chaperones are in limiting amounts, compared to soluble histones [43,44]. It is well known that Asf1-H3/H4 dimer are substrates for acetylation and deacetylation reactions that allow transference of the dimer to other chaperones or chromatin remodeling factors [45]. Indeed, histone H4 acetylation and deacetylation reactions occur during chromatin remodeling, when the H3/H4 associates with Asf1 [46]. Interestingly, we observed a decreased interaction with H4K10R decreased and an increased interaction with H4K14R in Asf1 pull down experiments. Such differential effect shows that chaperone interaction with H4 is variable for each acetylation. For example, H4K10Ac could be important for binding, while H4K14 for the release, both important to the dynamic formation of nucleosomes. Impairment of any one would disturb the cell causing an increase of free histones, as observed. As we measured the interaction with non-acetylatable H4, it would be important in the future to perform experiments with acetylated H4 in each position to definitively demonstrate that the effects observed in the present work are related to changes in the interaction with Asf1.

5. Conclusion

We concluded that acetylation of lysines 10 and 14 in the histone H4 have a role in the chromatin assembly and remodeling, affecting transcription and replication in trypanosomes. These acetylations are required for chromatin remodeling through modulation of histone interactions with Asf1 and other chaperones or chromatin remodeling components.

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