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# Histone Genes Expression during the Cell Cycle in *Trypanosoma cruzi*

**V. Sabaj,[1] L. Aslund,[2] U. Pettersson,[2] and N. Galanti[3]***

[1]Program of Cellular and Molecular Biology, and Program of Parasitology, Institute of Biomedical Sciences (ICBM), Faculty of Medicine, University of Chile, Santiago, Chile
[2]Department of Genetics and Pathology, Section of Medical Genetics, Rudbeck Laboratory, Uppsala, Sweden
[3]Program of Cellular and Molecular Biology, Institute of Biomedical Sciences (ICBM), Faculty of Medicine, University of Chile, Santiago, Chile

**Abstract** Histones, the basic proteins which compact DNA into the nucleosomal and solenoidal fibers are synthesized in correlation with DNA replication during the S-phase of the cell cycle. This behavior is controlled both at transcriptional and postranscriptional levels in higher eukaryotes and yeasts. We have found that histone synthesis in synchronized trypanosomes is controlled by fluctuations on the levels of their mRNAs. Though we cannot preclude the existence of a transcriptional regulatory mechanism, our results point to the participation of changes in the stability of histone mRNAs as a regulatory mechanism of their levels during the cell cycle in *Trypanosoma*. We have also found a postranscriptional regulatory mechanism which could be acting at the translational level. These results show both similarities and differences between *Trypanosoma* and higher eukaryotes regarding the expression of their histone genes. J. Cell. Biochem. 80:617–624, 2001. 

**Key words:** *Trypanosoma cruzi*; control of gene expression; histone messengers; cell cycle

In the nucleus of most eukaryotic cells DNA is coiled around an octamer of basic proteins called histones (two of each H2A, H2B, H3 and H4) in a structure named nucleosome. Adjacent to the entrance and exit of DNA in the octamer, a fifth protein, histone H1, is located. Chromatin compaction and function depends on the presence of these chromosomal proteins [Kornberg and Lorch, 1999].

Histone synthesis is tightly associated with the cell cycle, and occurs concomitantly with DNA replication during S-phase of the cell cycle [Jackson, 1985; Stein and Stein, 1984]. This finely regulated pattern of expression has been an attractive model for the study of mechanisms underlying the control of gene expression. In animal and yeast cells, it has been observed that the expression of histone genes is regulated both at transcriptional and postranscriptional levels. At the transcriptional level, histone genes are mainly transcribed during the S-phase of the cell cycle [Baumbach et al., 1987; Hereford et al., 1982; Palla et al., 1994]; at the postranscriptional level, it has been shown that histone messengers are stabilized during the S-phase of the cell cycle [Osley, 1991; Sittman et al., 1983]. A control at splicing and transport of the messenger to the cytoplasm has been also suggested [Luscher et al., 1985]. Both the coupling of histone synthesis with DNA replication and the mechanisms of control of this synthesis seem to be conserved during evolution.

Organisms of an ancient origin present some characteristics which seems aberrant when compared with those of more recent origin. Trypanosomatids are a good example: mRNAs transcribed from nuclear genes contain the miniexon, a common leader sequence on their 5′ end, which is postranscriptionally added via

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*Correspondence to: Norbel Galanti, Program of Cellular and Molecular Biology, Institute of Biomedical Sciences (ICBM), Faculty of Medicine, University of Chile, Santiago, Chile. E-mail: ngalanti@machi.med.uchile.cl

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transplicing [Vanhamme and Pays, 1995]; mRNAs originating from their kinetoplastic DNA are postranscriptionally modified by a mechanism known as RNA editing, in which nucleotides are added or deleted from the original transcript [Benne, 1994]; several nuclear genes are organized in large tandem clusters and transcribed as polycistronic units [Vanhamme and Pays, 1995]. As a result of this particular form of transcription, control of gene expression is exerted preferentially at the posttranscriptional level [Berberof et al., 1995; Gale et al., 1994; Graham 1995; Nozaki and Cross, 1995; Texeira et al., 1995]. For many genes, it has been found that protein synthesis is scarce in spite of the presence of high levels of their messengers [Tomas and Kelly, 1996].

We have already studied the synthesis of histone proteins in synchronized cells of *Trypanosoma cruzi*, and have found that is similar to what can be observed in modern eukaryotes: Core histones (H2A, H2B, H3 and H4) are synthesized exclusively during S-phase of the cell cycle, while synthesis of histone H1 is observed at a low rate all along the cell cycle, but highly increased during the S-phase [Sabaj et al., 1997]. In the present work we show that histone protein synthesis in synchronized trypanosomes is mainly, but only partially controlled by fluctuations on the levels of their RNA messengers. These fluctuations may be the result of regulatory mechanisms acting at the transcriptional level, however, our results point to the participation of changes in the stability of histone mRNAs as a regulatory step during the cell cycle in *Trypanosoma*. Furthermore, a postranscriptional mechanism seems to be present in the control of core histone synthesis, which could be acting at the translational level. Our results suggest, in accordance to what is known in trypanosomatids, that this postranscriptional control seems to be important in the regulation of the expression of histone genes. In this way, the synthesis of histone proteins is regulated in different manner in different organisms with the same result: synthesis of histone proteins during S-phase of the cell cycle.

## METHODS

### Cell Culture

*T. cruzi* epimastigotes (the replicative cellular form of *T. cruzi*), Tulahuén strain, were grown at 28°C in Diamond medium [Diamond, 1968], supplemented with 2.5% fetal calf serum [FCS], 7.5 μM hemin, 100 U penicillin, and 100 μg/ml streptomycin. Cultures were maintained in exponential growth through periodic passages into fresh medium.

### Synchronization Procedure

Synchronization of DNA replication in epimastigotes was performed as described [Galanti et al., 1994]. Briefly, cells at the 5th day of culture (logarithmic phase of growth) were incubated for 24 h in fresh medium containing 10% FCS plus 0.02 M hydroxyurea (HU). Following this treatment, cells were washed twice in phosphate-buffered saline [PBS] and suspended in fresh medium containing 10% FCS. Aliquots of cells were taken at different intervals. DNA synthesis was estimated by measuring [$^3$H]-thymidine incorporation into DNA [Sabaj et al., 1997].

### Inhibition of DNA Synthesis

Epimastigotes at the 5th day of culture were washed twice in PBS and incubated in fresh medium containing 10% FCS and 0.02 M HU. After 1, 2, 4 and 6 h in these conditions, aliquots of cells were washed and [$^3$H]-thymidine incorporation into DNA was measured. Two hours of HU treatment was chosen for the following assays. Cells without HU treatment were used as control.

### Inhibition of Protein Synthesis

After 2 h of incubation in the presence of 0.02 M HU, epimastigotes were divided into two aliquots. The first aliquot (G1 cells) was incubated for 20 min in cycloheximide 50 μg/ml, in the presence of HU. The second aliquot of cells was washed twice in PBS to eliminate the HU, and incubated for 6 h in fresh medium plus 10% FCS (S-phase cells). After this time, these S-phase cells were incubated for 20 min in 50 μg/ml cycloheximide. In both experiments, cells which were not treated with cycloheximide were used as control. In each case cells were washed twice in PBS, DNA synthesis estimated and total RNA purified.

### Histone Synthesis

Histone synthesis was quantified by incubating epimastigotes in [$^3$H]-lysine as described by Sabaj et al. [1997]. After incubation, histones were extracted from chromatin [Panyim and

Chalkey, 1969; Toro and Galanti, 1988; Toro and Galanti, 1990] and loaded on gels containing 15% polyacrylamide, 6 M urea, 0.9 N acetic acid, and 0.38% Triton DF-16. Electrophoresis was carried out as described by Toro and Galanti [1988]. After staining [Zweidler, 1980], gels were submerged for 30 min in Amplify (Amersham) and dried on a gel dryer under vacuum. Dried gels were placed in contact with hyperfilm MP (Kodak) and exposed for 1–2 months at −80°C.

### Total RNA Purification

$(1 \times 10^7)$ Cells were washed in PBS, resuspended in 1 ml of Trizol (GIBCO BRL) and incubated for 5 min at room temperature. About 200 μl of chloroform were then added to the tube, and after mixing, the sample was incubated at room temperature for 2–3 min and then centrifuged for 15 min at 10,000*g* at 4°C. The supernatant was transferred to an RNAse free tube and cold propanol was added; after 10 min at room temperature the samples were centrifuged for 10 min at 10,000*g*. The pellet containing the RNA was washed once in cold 75% ethanol, and centrifuged for 5 min at 7,500*g*. After drying, the pellet was suspended in diethylpyrocarbonate (DEPC) treated water.

### Northern Blots

Ten or twenty micrograms of total RNA were loaded on 1% agarose, 2.2 M formaldehyde gels and run for approximately 6 h at 75 V. Total RNA was transferred from the agarose gels to nylon membranes (Hybond $N^+$, Amersham) as described by Sambrook et al. [1982]. Membranes were air-dried and the RNA was immobilized by heat (80°C) for 2 h.

After washing, membranes were incubated in prehybridization solution (formamide 50%, 5 × SSC, 50 mM sodium phosphate pH 6.5, 5 × Denhardt's solution, 0.1% SDS and 100 μg/ml salmon sperm DNA) for 4 h at 42°C. Probes were radioactively labeled with dCTP-[αP32] by random priming (Oligolabelling kit, Pharmacia) and added to the prehybridization solution. Membranes were incubated with the hybridization solution at 42°C overnight. After hybridization, membranes were washed three times, for 30 min, at 65°C in 2 × SSC, 0.5% SDS, once in 0.1 × SSC, 0.1% SDS at 65°C and film exposed (Kodak X-Omat S) for 12 h at −80°C.

### Probes

Probes for H2A and H2B were obtained by PCR amplification of *T. cruzi* genomic DNA with primers designed from the published sequences [García-Salcedo et al., 1994; Puerta et al., 1994], and consisted of 400 and 300 bp segments of the coding region, respectively. H3 probe was a 1,000 bp segment from a *T. cruzi* genomic clone including 3′ end of the gene, a space region and a 5′ end of the next gene of the tandem (kindly provided by E. Bontempi). The H4 probe was a 250 bp PCR amplified fragment from the *T. cruzi* histone H4 gene (Aslund, Genbank Accession AI078938). H1 probe was a 300 bp segment of the coding region of H1 gene from a cDNA clone [Aslund et al., 1994].

## RESULTS

Epimastigotes of *T. cruzi* on the 5th day of culture were treated with 0.02 M hydroxyurea (HU) for 24 h in order to synchronize their DNA replication. At the end of HU treatment (0 h) and at 6, 12 and 24 h after recovery of HU treatment, aliquots of cells were taken, and histone synthesis and levels of histone messengers were determined. Section B of Figure 1 shows that an increase in the synthesis of core histones H2A, H2B, H3 and H4 is observed only at 6 and 12 h (lanes 3 and 4, respectively), when DNA replication is active (Fig. 2, line). Northern blots hybridized with H2A, H2B, H3, and H4 probes, at 0, 6, 12 and 24 h after HU treatment are shown in section A of Figure 1 (lanes 2, 3, 4 and 5, respectively). RNA purified from epimastigotes at the 5th day of culture, previous to HU treatment, was loaded as control on lane 1. All these histone mRNAs showed similar fluctuations, decreasing their steady state levels after incubation of the cells in HU (compare lanes 1 and 2 of Fig. 1A and see Fig. 2). Six hours after the release of HU blockade, histone mRNAs levels increased considerably (Fig. 1A, lane 3 and Fig. 2). At 12 h, histone messenger levels were still high (Fig. 1A, lane 4 and Fig. 2), and at 24 h after the release of the blockade, levels were near to those of control epimastigotes (compare lanes 1 and 5 of Fig. 1A and Fig. 2). During S-phase (6 and 12 h after HU washout), when core histones are synthesized, levels of their messengers increased, which suggest that variations in core histone synthesis during the cell cycle is regulated at

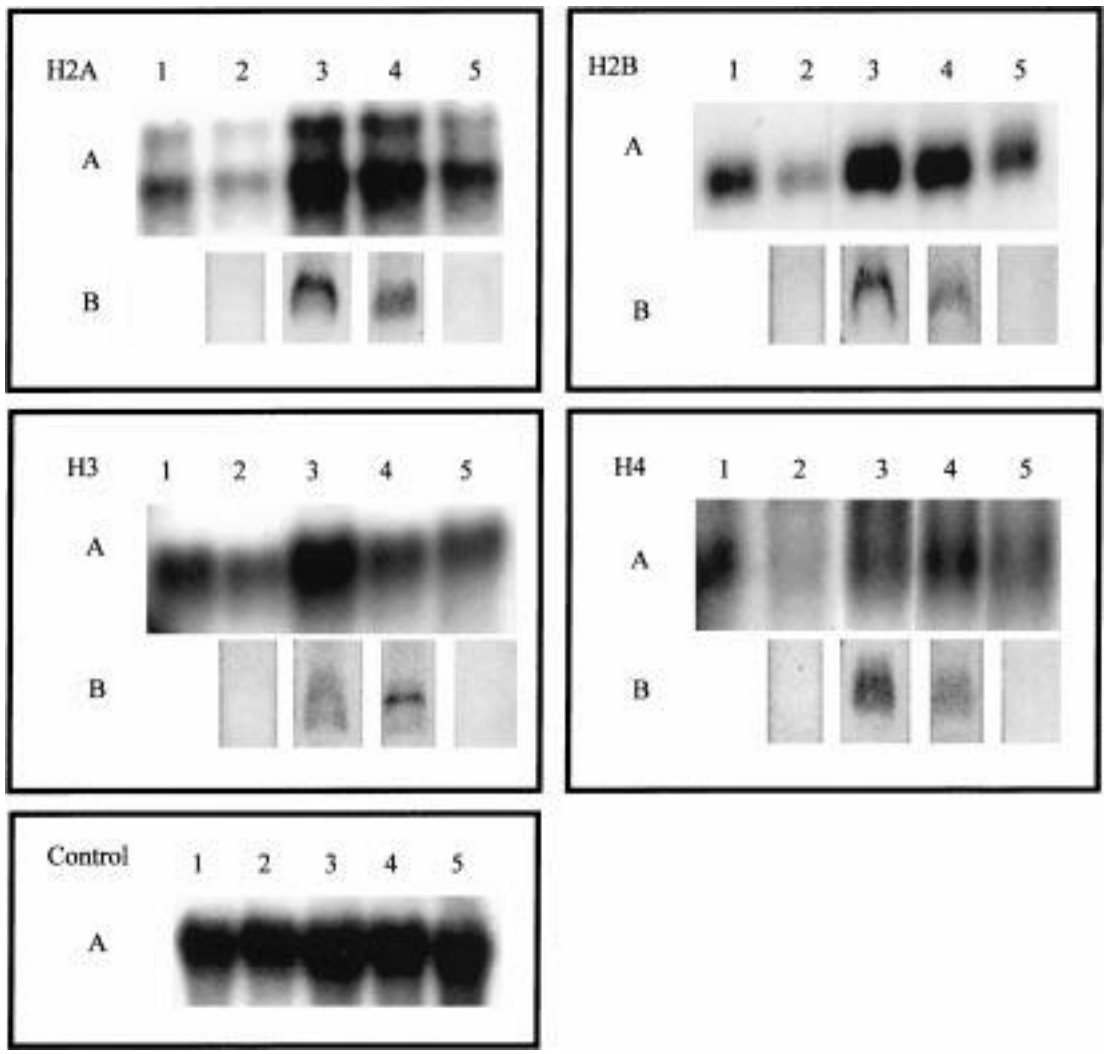


**Fig. 1.** Core histones expression along the cell cycle in synchronized epimastigotes. (**A**) Northern blot assays: Total RNA (20 μg) from asynchronic epimastigotes at the 5th day of culture (lane 1) and synchronized epimastigotes at 0, 6, 12 and 24 h after HU washout (lanes 2, 3, 4 and 5) was separated on a 2.2 M formaldehyde, 1% agarose gel, transferred to nylon membranes and hybridized to $^{32}$P-labeled core histone DNA probes. Equivalent loading was confirmed based on ribosomal RNA levels as reflected by ethidium bromide staining of gels; appropriate transfer was checked by using a constitutive cystein proteinase gene as probe (Control A). (**B**) Protein synthesis: epimastigotes treated as in A, were incubated with $^{3}$H-lysine for 3 h. Histones were purified and separated in TAU-gels. After dried, gels were exposed.

their mRNA levels. The results also suggest that this is not the only level at which control of the expression of these genes is exerted, because core histone mRNAs were detected when these proteins are not being synthesized (0 and 24 h after HU washout, see Figure 1, lanes 2 and 5, and Fig. 2), which could be explained by a control of gene expression at the postranscriptional level.

Figure 3B shows synthesis of histone H1 at all the studied times, although it is highly increased at 6 and 12 h after HU washout (lanes 2 and 3, respectively), when DNA is being actively synthesized. Northern blots hybridized to an H1 probe demonstrated that changes of the mRNA levels closely parallels the variation of protein synthesis, showing an increase at 6 and 12 h (Fig. 3A, lanes 2 and 3). This parallelism indicates that variations of histone H1 synthesis are controlled at the level of abundance of its messengers.

From time ago it has been observed that protein synthesis inhibition prevents histone mRNAs decrease in cells in which DNA replication was inhibited with HU [Baumbach et al., 1984]. Accordingly, protein synthesis was

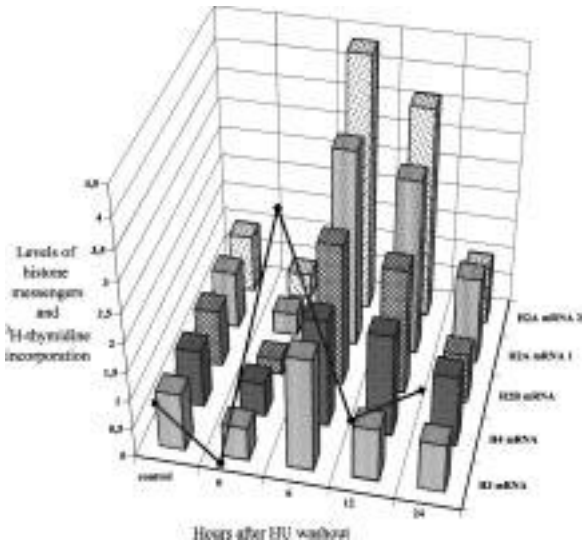


**Fig. 2.** Variations of core histone mRNA levels along the cell cycle in HU synchronized epimastigotes. Columns: Northern blots showed in Figure 1 were scanned and the OD for each core histone messenger is expressed relative to the OD measured in asynchronic epimastigotes (control). Line: $^{3}$H-thymidine incorporation into DNA was also standardized according to its incorporation in asynchronic epimastigotes. 0, 6, 12 and 24 refers to hours after HU washout.

inhibited in HU-treated *T. cruzi* epimastigotes. Firstly, we looked at the shortest time of DNA synthesis inhibition after treatment with HU, and found that 2 h after addition of this drug, DNA synthesis was inhibited by 90% (not shown). Afterwards, cells treated for 2 h in the presence of HU (G1 cells), as well as epimastigotes 6 h after releasing the blockade of HU (S-phase cells) were incubated for 20 min with cycloheximide. This drug effectively decreased $^{3}$H-leucine incorporation into total proteins (not shown). Cells without cycloheximide treatment were used as control in each case. Variations of mRNAs levels as a result of protein inhibition are shown in Figure 4A for histone H3. Cycloheximide treatment increased the levels of these histone messengers both in cells in which DNA replication was inhibited (compare lanes 2 and 3) and in cells with an active DNA replication (compare lanes 4 and 5). The other nucleosomal histones behaved in a similar way (not shown). Figure 4B (columns) shows fluctuations of histone H3 mRNA in response to protein inhibition; in the same figure $^{3}$H-thymidine incorporation into DNA in these assays is shown (line). Accordingly, the stability of histone mRNAs seems to be also involved in the regulation of their levels during the cell cycle in *T. cruzi* epimastigotes.

## DISCUSSION

In the Trypanosomatids genome genes are clustered and co-transcribed in polycistronic units from a common promoter located on the 5′ end of each cluster. The main consequence of this organization is that the regulation of a particular gene on the cluster cannot be made at its transcriptional initiation. Accordingly, mechanisms of control of gene expression acting at a posttranscriptional level become relevant in these ancient eukaryotes [Graham, 1995; Vanhamme and Pays, 1995].

As showed in this report, all core histone messengers present similar variations along the cell cycle in synchronized epimastigotes of *T. cruzi*. Histone genes forming part of the same polycistronic unit, hints the possibility of a single step regulation, at the transcriptional level. Nevertheless, this is not the case, described histone genes in *Trypanosoma* are present on different chromosomal bands of PFGE [García-Salcedo et al., 1994; Henriksson et al., 1995; Puerta et al., 1994]. The location of histone genes in different transcriptional units implies they are transcribed at similar rates as all the genes present in that unit. The transcription at these different units can be assumed to be diverse, giving place to the accumulation of uneven amounts of the histone transcripts. Under these circumstances, posttranscriptional regulation becomes an important level of control.

Our results showed that levels of core nucleosomal histone mRNAs vary during the cell cycle in synchronized epimastigotes, reaching a maximum at the S-phase in coordination with DNA replication, as is the rule in eukaryotes of recent origin [Baumbach et al., 1987; Osley, 1991; Stein and Stein, 1984]. These results confirmed and extended recent data on the levels of particular nucleosomal histone mRNAs in logarithmic and in stationary growth phase trypanosomatids [García-Salcedo et al., 1994; Marañon et al., 1998; Soto et al., 1996, 1997].

We have previously shown that nucleosomal histone protein synthesis varies during the cell cycle in DNA replication synchronized epimastigotes, in close correlation with DNA replication [Sabaj et al., 1997]. In this report, we show that these fluctuations in nucleosomal histone

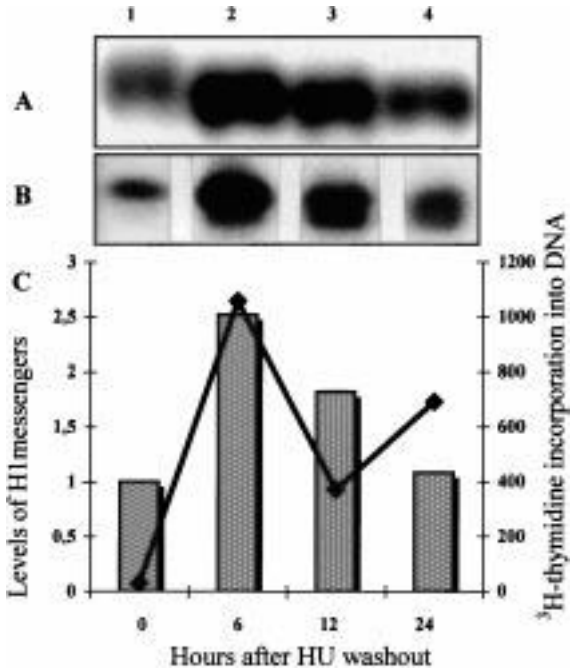


**Fig. 3.** H1 histone expression along the cell cycle in synchronized epimastigotes. (**A**) Northern blot assays: total RNA (20 µg) from synchronized epimastigotes at 0, 6, 12 and 24 h after HU washout (lanes 1, 2, 3 and 4) was separated on a 2.2 M formaldehyde, 1% agarose gel, transferred to a nylon membrane and hybridized to $^{32}$P-labeled H1 histone probe. Equivalent loading and transfer were confirmed as indicated under Figure 1. (**B**) H1 protein synthesis: epimastigotes treated as in A, were incubated with $^3$H-lysine for 3 h. Histones were purified and separated in TAU-gels. After dried, gels were exposed. (**C**) Columns: Northern blots shown in A were scanned and the OD is expressed relative to OD measured in cells at the end of HU treatment (0 h). Line: $^3$H-thymidine incorporation into DNA expressed as cpm $\times 10^{-1}$ per million of cells.

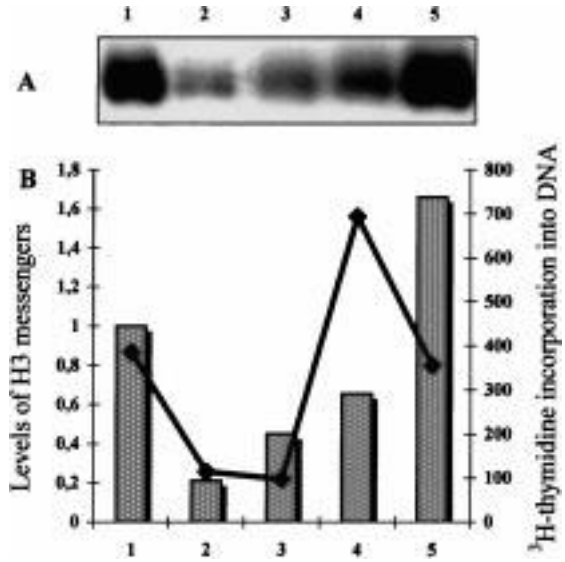


**Fig. 4.** H3 histone messenger variations in response to cycloheximide. (**A**) Norther-blot assay: total RNA (20 µg) was extracted from epimastigotes at the 5th day of culture (control, lane 1), from epimastigotes treated 2 h with HU (lane 2), from epimastigotes treated 2 h with HU and 20 min with cycloheximide (lane 3), from epimastigotes treated with HU and incubated in fresh medium plus 10% FBS for 6 h after HU washout (lane 4) and from epimastigotes treated with HU and incubated in fresh medium plus 10% FBS for 6 h after HU washout and then incubated 20 min in cycloheximide (lane 5). These RNAs were separated on a 2.2 M formaldehyde, 1% agarose gel, transferred to a nylon membrane and hybridized to $^{32}$P-labeled H3 histone probe. Equivalent loading and transfer were confirmed as indicated under Figure 1. (**B**) Columns: Northern blots shown in A were scanned and the OD expressed relative to OD measured in cells at the 5th day of culture (control). Line: $^3$H-thymidine incorporation into DNA expressed as cpm per million of cells.

protein synthesis may be explained, at least partially, by variations in the levels of their mRNAs, which sharply increase during DNA replication. A similar response is observed in eukaryotes of recent origin, in which variations of core nucleosomal histone mRNAs are the result of both an increased transcription of these histone genes and the increased stability of their mRNAs during DNA replication, which become unstable outside the S-phase [Sittman et al., 1983; Stein and Stein, 1984; Osley, 1991]. However, we have found important levels of nucleosomal histone mRNAs in *T. cruzi* epimastigotes when their DNA replication is inhibited with HU, as well as when the cells enter the G2 phase of the cell cycle [18–24 h after HU washout, Galanti et al. 1994]. These results suggest that mRNAs of nucleosomal histones are more stable in *T. cruzi* than in eukaryotes of recent origin. In these organisms, the decrease in stability of histone mRNAs when DNA replication is inhibited depends on active protein synthesis. Accordingly, we have found that inhibition of protein synthesis with cycloheximide moderately increases the level of nucleosomal histone mRNAs in *T. cruzi* epimastigotes when their DNA replication is also inhibited. Nevertheless, this increase is far from the levels of nucleosomal histone mRNAs present in cells at the S-phase of the cell cycle, which suggests that these messengers are under the control of some other mechanism besides their stabilization during DNA replication.

Surprisingly, the levels of nucleosomal histone mRNAs are sharply increased in cyclohex-

imide treated cells when they are in active DNA replication (log cells). This result supports the idea of a more unstable nucleosomal histone mRNAs in *T. cruzi* during DNA replication, contrary to what it was found in more recent eukaryotes.

Taking all these data into consideration, our results strongly suggest that the stability of histone mRNAs plays an important role as a regulatory mechanism of their levels, during the cell cycle in *T. cruzi* epimastigotes. However, our results do not preclude the participation of a transcriptional regulatory mechanism to explain the observed changes in the levels of histone mRNAs in these organisms.

Interestingly, no synthesis of nucleosomal protein histones was found when DNA replication was inhibited, or in G2-phase epimastigotes, although their messenger RNAs were still present. This result points to a level of control that is not transcriptional, but postranscriptional. Postranscriptional regulation of histone genes could be exerted by nuclear retention of immature messengers and by translational control. Our results do not discriminate between these alternatives, but we favor translational control for two reasons. Firstly, we only observe nucleosomal histone mRNAs of the expected length in Northern blots; longer messengers should be expected in case of nuclear retention of not spliced messengers. Secondly, translational control is a frequently reported level of regulation of gene expression in Trypanosomatids [Nozaki and Cross, 1995; Texeira et al., 1995].

Regarding H1 mRNAs, their levels seem to fluctuate in parallel with those of nucleosomal mRNAs during the cell cycle in *T. cruzi*. However, histone H1 protein synthesis occurs when DNA replication is blocked by hydroxyurea, as well as at G2-phase cells, when nucleosomal proteins are not synthesized, pointing to a difference in the control of nucleosomal histones and H1 histone expression. Thus, H1 protein synthesis in *T. cruzi* cell cycle seems to be similar to the one described in higher eukaryotes.

In recent eukaryotes the destabilization of the messengers observed out of the S-phase, relies on a stem-loop structure located at the 3′UTR region [Graves et al., 1987; Levine et al., 1987], which is also necessary for the observed effect of cycloheximide on messenger levels [Pandey and Marzluff, 1987]. In Trypanosomatids, on the other side, hairpin structures at the 3′UTR of the messengers have been reported to participate on the regulation of the expression of genes at the transcriptional level [Hehl et al., 1994; Nozaki and Cross, 1995]. Looking for these kind of structures in histone messengers in *T. cruzi*, we have found possible areas where hairpin could be formed in 3′UTR of H2A, H3 and H1. In view of the results presented in this report, hairpins on these messengers could be acting at any one of the two levels described; determining the half life of the messengers and regulating the ability of the messengers to be translated in a cell cycle dependent form.

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