Characterization of anti-silencing factor 1 in *Leishmania major*

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Anti-silencing factor 1 (ASF1) is a histone chaperone that contributes to the histone deposition during nucleosome assembly in newly replicated DNA. It is involved in chromatin disassembly, transcription activation and in the cellular response to DNA damage. In Leishmania major *the ASF1 gene (*LmASF1*) is located in chromosome 20 and codes for a protein showing 67% of identity with the* Trypanosoma brucei *TbASF1a. Compared to orthologous proteins,* LmASF1 *conserves the main residues relevant for its various biological functions. To study ASF1 in* Leishmania *we generated a mutant overexpressing* LmASF1 *in* L. major*. We observed that the excess of* LmASF1 *impaired promastigotes growth rates and had no impact on cell cycle progress. Differently from yeast, ASF1 overproduction in* Leishmania *did not affect expression levels of genes located on telomeres, but led to an upregulation of proteins involved in chromatin remodelling and physiological stress, such as heat shock proteins, oxidoreductase activity and proteolysis. In addition, we observed that* LmASF1 *mutant is more susceptible to the DNA damaging agent, methyl methane sulphonate, than the control line. Therefore, our study suggests that ASF1 from* Leishmania *pertains to the chromatin remodelling machinery of the parasite and acts on its response to DNA damage.*

Key words: *Leishmania major* - anti-silencing factor 1 - histone chaperone

Leishmania spp is a member of the Trypanosomatidae family and a parasitic protozoa and digenetic eukaryote of medical and veterinary relevance. *Leishmania* undergoes several changes to adapt to and survive in different hostile environments, namely the insect vector digestive tract and the vertebrate host's macrophagic phagolysosomes. For that a tight and agile control of gene expression is required.

Differently from most of the eukaryotes, these parasites have polycistronic transcription and control of gene expression occurs primarily at the posttranscriptional level (reviewed by Clayton 2002, Martinez-Calvillo et al. 2010). Likewise other eukaryotes, in these parasites the chromatin structure modulates the access of proteins to the DNA, which ultimately regulates different aspects of gene expression, DNA processing, replication and double-stranded DNA repair (Navarro et al. 1999, Elias et al. 2001, Gontijo et al. 2003, McNairn & Gilbert 2003).

Anti-silencing factor 1 (ASF1) is a histone chaperone that together with chromatin assembly factor (CAF)-1 adds histones H3 and H4 onto newly replicated DNA (Tyler et al. 2001). This suggests a role for ASF1 in chromatin assembly regulation (Loyola & Almouzni 2004, Zhang et al. 2005). ASF1 can be involved either in gene activation or transcriptional repression; it depends on the factors it interacts with (Sutton et al. 2001, Schwabish & Struhl 2006, Adkins et al. 2007, Takahata et al. 2009, Varv et al. 2010). In different eukaryotes deletion of ASF1 alters response to DNA damage and DNA replication blocking agents (Le et al. 1997, Tyler et al. 1999) and leads to gross chromosomal rearrangements (Prado et al. 2004) and even to cell death (Sanematsu et al. 2006).

The increased susceptibility of chromatin-assembly factor mutants to DNA-damaging agents may be an effect of the direct role of these factors in modulating chromatin structure (Mello et al. 2002). In addition, an association between ASF1 and genomic stability has been provided by the identification of a dynamic interaction between ASF1 and the Rad53 DNA damage checkpoint protein. Also, activation of ASF1 may be an important cellular response to DNA damage and replication stress (Emili et al. 2001, Hu et al. 2001). When DNA damage occurs, ASF1 is recruited to the lesion site where it may disrupt the H3/H4 tetramer. This results in nucleosome eviction and allows access of the repair machinery to unencumbered DNA (Canfield et al. 2009).

In *Leishmania* species ASF1 roles have not been investigated, although orthologous genes have been annotated in the sequenced genomes (tritrypdb.org). In another trypanosomatid, *Trypanosoma brucei*, the participation of ASF1 in the machinery that regulates spindle assembly and S-phase progression has been described (Li et al. 2007). Therefore, given that ASF1 is associated with gene expression in other eukaryotes and that chromatin modifications are relevant for controlling

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transcription in trypanosomatids (Green & Almouzni 2003, Figueiredo et al. 2009) we investigated *Leishmania major*'s ASF1. Our aim was to understand which of the known functions of ASF1 from other eukaryotes are shared by this parasite's protein.

MATERIALS AND METHODS

Parasites culture and transfection - L. major MHOM/IR/83/IR (LmCC-1) promastigotes were grown at 26ºC in M199 medium supplemented with 10% heat inactivated foetal calf serum, HEPES (0.04 M), adenine (0.1 mM), biotin (0.1 %), haemin (0.25 %), penicillin (2.5 U/mL^{-1}), streptomycin (2.5 mg/mL^{-1}). Transfection experiments were conducted as described elsewhere (Kapler et al. 1990). Mutants overexpressing ASF1, Lm*[pX63NEO-ASF1]* and Lm*[pX63NEO],* which bears the vector with no insert, were selected and cultured in the presence of G418. Increasing concentration of drug of selection (G418) was used to raise plasmid copy number and obtain higher levels of expression of episomal genes. G418 LD_{50} was determined (1 µg.mL^{-1}); we worked on a range of two-100 folds the LD_{50}.

Plasmid constructs - A 1,246 bp fragment from pUCE8*HH*5.4, containing *LmASF1* ORF and its 5' and 3'UTR, was obtained after *Aat*II digestion. This plasmid was constructed with a 5.4 kb *Hin*dIII fragment subcloned from a cosmid, it has been described elsewhere (Pedrosa et al. 2001). pX63NEO-ASF1, which was used to overexpress the *LmASF1* in *L. major*, was obtained by insertion of the 1,246 bp fragment into pX63NEO *Aat*II-digested (LeBowitz et al. 1991).

RNA extraction and northern blotting hybridization - RNA from *L. major* in vitro cultures was extracted with Trizol® reagent following the manufacturer's protocol. Twenty micrograms of total RNA of each sample were fractionated on 2.2 M formaldehyde 1.2% agarose gel and transferred to a hybond-N+ nylon membrane. Subsequent steps for probe labelling and northern hybridization were conducted according to established protocols (Feinberg & Vogelstein 1983).

Flow cytometry analysis and propidium iodide staining - L. major control and ASF1 overexpressor cells were prepared according to Shapiro (Shapiro et al. 1984). The DNA content of PI-stained cells was analyzed with a FACScan analytical flow cytometer using the Cellquest software (BD Biosystems).

Total protein cell extracts and two-dimensional gel electrophoresis (2D) - Cells (1 x 10^9) in late-log phase of axenic growth were pelleted (2,000 *g*/10 min/4ºC), washed twice with 1x phosphate buffered saline (PBS) (pH 7.0) and ressuspended in 1 mL 0.1x PBS containing protease inhibitors (Complete, Roche) for cell lysis. Cell extracts were submitted to five freeze-thaw cycles in liquid N_2/water bath at 30ºC and cellular debris were removed by two rounds of centrifugation (10,000 *g*/10 min, at 4ºC). The supernatants were precipitated with 5 mL 0.1x PBS and trichloroacetic acid (TCA) at 4ºC for 2 h. Precipitates were collected by centrifugation at 10,000 *g* followed by two washes with frozen acetone. Proteins were solubilised in 300 µL of rehydration buffer [7 M urea, 2 M thiourea, 4% CHAPS (w/v)] plus nuclease solution (160 units of DNAse and 125 µg RNAse) and 10 mM $MgCl_2$ and incubated for 12 h at room temperature. Protein concentration was determined by the Bradford method (Bradford 1976).

Independent cultures of Lm*[pX63NEO]* and Lm*[pX63NEO-ASF1]* were used for comparative analysis by 2D gel electrophoresis Immobiline DryStrip gels (13 cm: 4-7, GE-HealthCare) were rehydrated in 700 µg of parasite protein sample. Isoelectric focusing (IEF) was carried out at 20ºC with maximum current setting at 50 µA per strip, using an active two-step rehydration system of 2 h at 0 V and of 10 h at 30 V, directly in ceramic strip holders, and then focused on an IPGphor IEF unit (GE HealthCare). Conditions for IEF were: 1 h at 500 V, 1 h at 1,000 V, 1 h at 2,000 V, 1 h at 4,000 and at 8,000 V until voltage reached 55,000 Vh. Focused pH gradient strips were then fractionated in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% polyacrylamide) according to the GE-HealthCare manual. Gel fixation and colloidal Coomassie staining were performed according to (Neuhoff et al. 1988). Gels were stored at 4ºC in 25% ammonium sulfate and scanned with ImageScanner III (GE-HealthCare). Comparative analysis of digitized proteome maps was conducted with ImageMaster Platinum v7.0 software (GE-HealthCare). Differences higher than one-and-a-half-fold in the pattern and/or intensity of protein spots of analyzed strains were identified by pairwise comparison using gel images (Supplementary data). The proteins were identified by mass spectrometry using a MALDI TOF-TOF (4700 Proteomics Analyzer, Applied Biosystems).

Real-time PCR (RT-PCR) - Relative quantification - RT-PCR was performed using an ABI 7500 Sequence Detection System (Applied-Biosystems) in the presence of SYBR-green. The optimization of the RT-PCR reaction was performed according to the manufacturer's instructions (Applied-Biosystems, User Bulletin 2 applied to the SYBR-Green I core reagent protocol). The reactions were performed as technical and biological triplicates using SYBR Green (SYBRGreen PCR Master Mix, Applied Biosystems). Final concentration of oligonucleotides in the reaction was 200 nM and the identification of the 44 targeted transcripts and primers sequences are provided as Supplementary data. GLucose-6-phosphate dehydrogenase was used as the control of constitutive gene expression. To evaluate the efficiency of amplification reaction of each gene-target, a standard curve was generated using 10-fold dilutions of cDNA. The relative expression was analyzed by $2^{-\Delta\Delta CT}$ method.

Production of polyclonal antiserum anti-ASF1 and western blotting analysis - Rabbit serum was obtained by immunization with a recombinant polypeptide corresponding to the *T. brucei* ASF1 gene. This was PCR amplified using Asf-FowNdeI (5'-CATATGAGATACAACCAATT) and Asf-RevBamHI (5'-GGATCCTCATCTGGGTTCAAGTGC) primers from the parasite genomic DNA and cloned in *Nde*I-*Bam*HI sites pET14b.

After induction with 0.5 mM IPTG for 12 h at 37ºC, the recombinant protein was extracted with BugBuster Protein Extraction Reagent (Novagen) and purified by affinity chromatography on Ni-Agarose (Qiagen). Specific antibodies were purified with the same ASF1 used for the immunization. The recombinant protein was immobilized in a cyanogen bromide-agarose beads (GE Healthcare) and after washes with 10 column volumes it has been eluted with 0.1 M triethylamine, pH 11. The fractions were neutralized to pH 8 with Tris-HCl 1 M pH 7.4 and dialyzed against PBS.

For western blots, total protein was extracted from 1 x 10^7 parasites. Samples were fractionated in 12.5% SDS-PAGE, transferred to nitrocellulose membranes. Total proteins were visualized by staining with Ponceau-S and incubated with primary antibody in blocking solution. Antibody dilutions used were 1:500 to 1:1,000. The membrane was washed three times for 10 min with PBS, 0.1% Tween-20 and incubated with protein A 1:10,000 or anti-rabbit IgG (1:20,000) both conjugated to HRP (Invitrogen) for 45 min. The membrane was treated and bond antibodies developed following ECL kit instructions (ECL, Millipore).

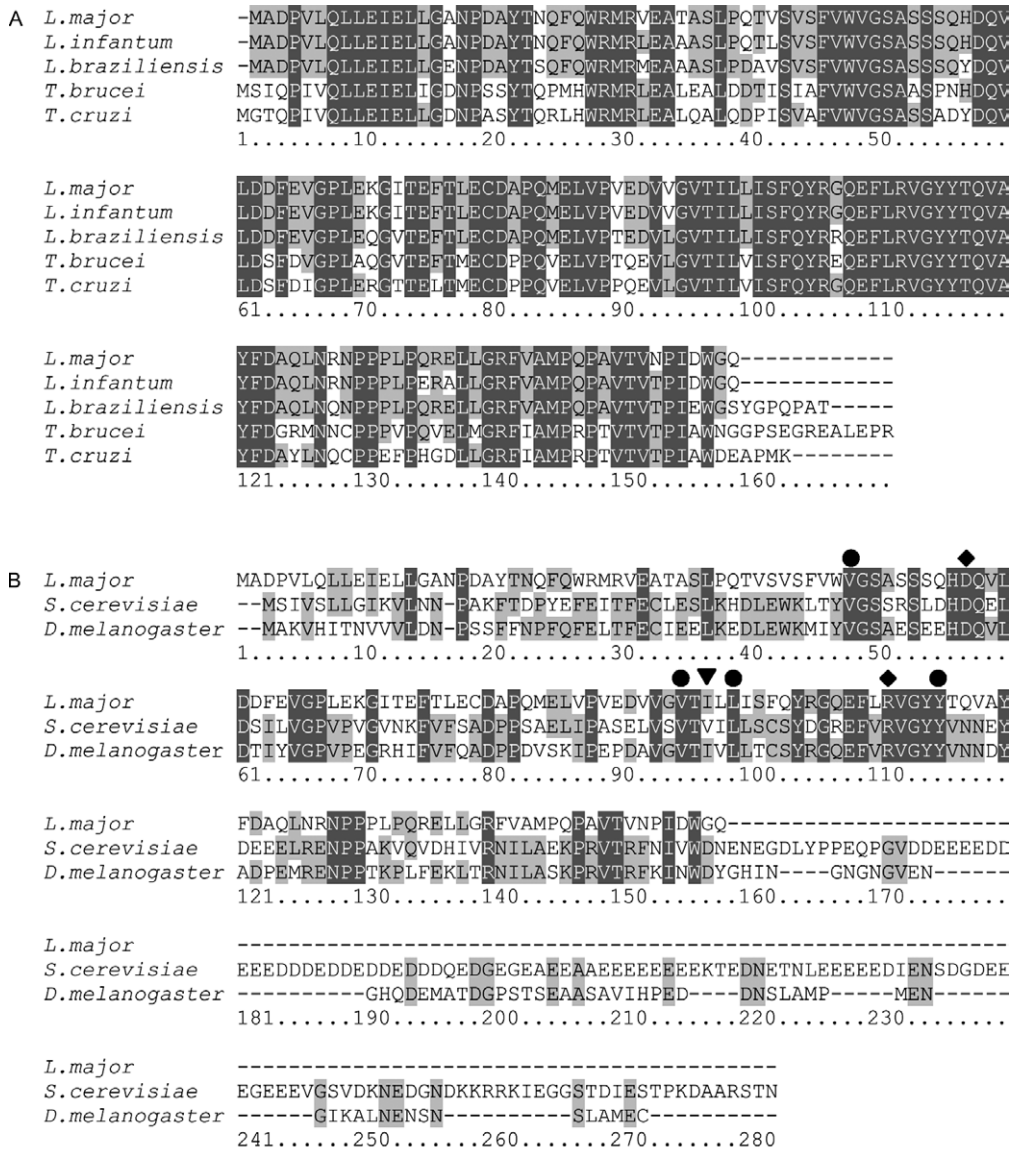

Fig. 1: conservation of anti-silencing factor 1 (ASF1) sequence. A: alignment of ASF1 from Trypanosomatidae showing high conservation in aminoacid residues (~67%); B: alignment of *Leishmania major, Saccharomyces cerevisiae* and *Drosophila melanogaster* ASF1 showing conserved residues of N-terminal region. Identical residues are shaded dark grey while conserved residues are shaded light grey. The symbol (•) means the amino acids V45, V92, L96 and Y112 and (♦) means the residues D54 and R108. The substitution V94I is indicated by the arrow head. The accessions of ASF1 sequences are: *L. major*, AAK28282.1, *Leishmania infantum*, CAM67383.1, *Leishmania braziliensis*, XP_001564694.1, *Trypanosoma brucei*, CAJ15961.1, *Trypanosoma cruzi*, XM_810961.1, *S. cerevisiae*, AAC37512.1 and *D. melanogaster*, AAF15354.1. The aminoacid positions correspond to *D. melanogaster* ASF1 sequence.

Western blot signal quantification by ImageJ - We selected the band indicated by "*LmASF1*" (at the right end of panel C) for quantification. ImageJ square tool was selected and used to determine the number of pixels per band using the tool named "plot profile". The number of pixels obtained per band was subtracted from background pixels. The relative expression was calculated by dividing the value obtained for the signal detected in the transfectant by that observed in the control strain. Normalization of the values was conducted using the corresponding Ponceau-stained membrane.

RESULTS

L. major ASF1 sequence conservation - The ASF1 primary sequence is highly conserved throughout evolution, as it is shown by the in silico comparative analysis of the ASF1 homologues among trypanosomatids, *Saccharomyces cerevisiae* and *Drosophila melanogaster* (Fig. 1A, 1B). *L. major* ASF1 (LmjF20.0050) comprises 158 amino acids and displays 67% sequence identity with the *T. brucei* ASF1a (Tb927.1.630). The N-terminal domain is highly conserved between *Leishmania, S. cerevisiae* and *D. melanogaster*, but BLAST analysis revealed that the ASF1 C-terminal region from trypanosomatids lacks the long poly-acidic tract found in yeast (Fig. 1B).

Effects of LmASF1 overexpression over growth rate and cell cycle - The detection of *LmASF1* transcript in promastigotes by northern blotting was only possible when at least 20 µg of total RNA was used, indicating that its endogenous level is low. We observed that the *LmASF1* transfectant (Lm*[pX63NEO-ASF1]*) has higher levels of *LmASF1* transcript as compared to other transcripts. A positive correlation between drug concentration and increment of transcript levels was detected (Fig. 2A). The larger RNAs hybridizing with the ASF1 probe are probably read-through transcripts, commonly found in episomal transcription in *Leishmania*. Relative expression of the *LmASF1* transcript in log phase promastigotes is 90.5 times higher in the overexpressor as compared to the control line (Fig. 2B). Interestingly, the protein level seeing as 18 KDa band detected by western blot using an anti-*T. brucei* ASF1 increased, but the increment did not seem so high (Fig. 2C). Image quantification revealed that ASF1 was at least 3.0 fold augmented in the overexpressor as compared to the control line Lm*[pX63NEO].* Quantification was conducted with the western band signals from cells kept at 100 x LD_{50} to be comparable to the quantitative RT-PCR estimation of the transcript (Fig. 2C).

In addition overexpression of *LmASF1* decreased slightly the growth rate of promastigotes in axenic culture (Fig. 3A). The doubling time of the overexpressor mutant is 8.67 h as compared to 6.84 h of the control line. We did not witness significant changes of the cell cycle progression after submitting non-synchronized populations of ASF1 overexpressor as compared with the control transfectants (Fig. 3B).

Changes in gene expression pattern in the LmASF1 overexpressor - Because the *S. cerevisiae's* ASF1 is an ASF that releases the expression of genes located at the very end of the chromosomes, we investigated whether a similar role is played by its *L. major* orthologue, the *LmASF1*. We evaluated the transcript levels of 42 genes located in various chromosomes by comparing Lm*[pX63NEO-ASF1]* and Lm*[pX63NEO]*, most of them from the chromosomes' extremities (see distance of each gene from its telomeric end) (Table I). We found that expression of only five genes was affected by ASF1 overexpression (Table I). Among the few modulated genes, only one, the second annotated gene on chromosome 4 (LmjF04.0020), is less than 16 kb far from a telomere, as shown on Table I.

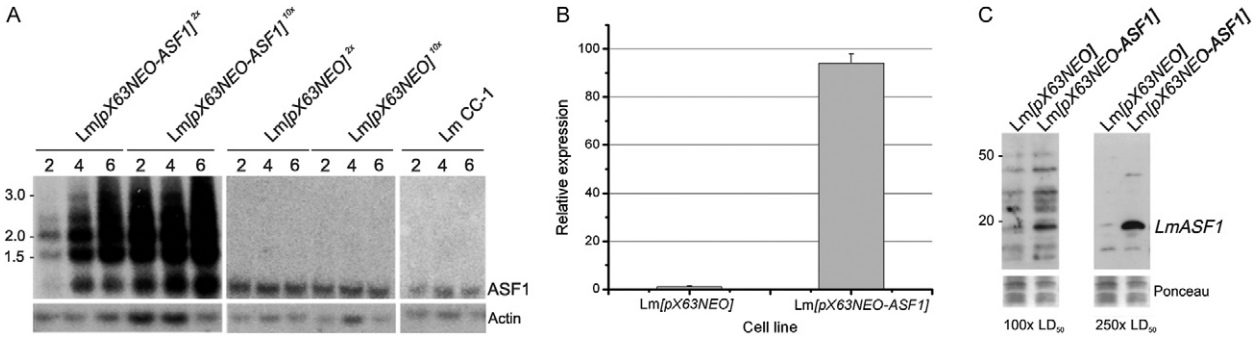

Fig. 2: anti-silencing factor 1 (ASF1) overexpression in *Leishmania major.* A: total RNA was extracted from promastigotes after two, four and six days in axenic culture (at the top of panel A: 2, 4 and 6, respectively) and 20 µg was fractionated in denaturing agarose gel, transferred to nylon membranes and hybridized with radiolabeled ASF1 probe. The left panel shows increasing amounts of *LmASF1* transcript in log phase overexpressor in two conditions of drug pressure (Lm*[pX63NEO-ASF1]*^{2x} and Lm*[pX63NEO-ASF1]*^{10x}) as compared to control lines (control transfectant Lm*[pX63NEO]*^{2x}, Lm*[pX63NEO]*^{10x} and LmCC-1) which are presented in mid and right panels, respectively. A fragment of actin gene was radiolabeled and used as a control of RNA loading (strip shown at the bottom of panels). Numbers shown in superscript mode refer to the drug pressure used in each experiment (times the LD_{50} of G418); B: relative expression of *LmASF1* revealed by real-time polymerase chain reaction. The parasites were kept in media containing 100x LD_{50} of G418; C: immunoblotting of cell extracts from promastigotes of *L. major* overexpressor, Lm*[pX63NEO-ASF1],* and control line Lm*[pX63NEO]* in two conditions of drug pressure (100x LD_{50}, left panel and 250x LD_{50}, right panel) was probed with affinity purified antiserun to *Trypanosoma brucei* ASF1.

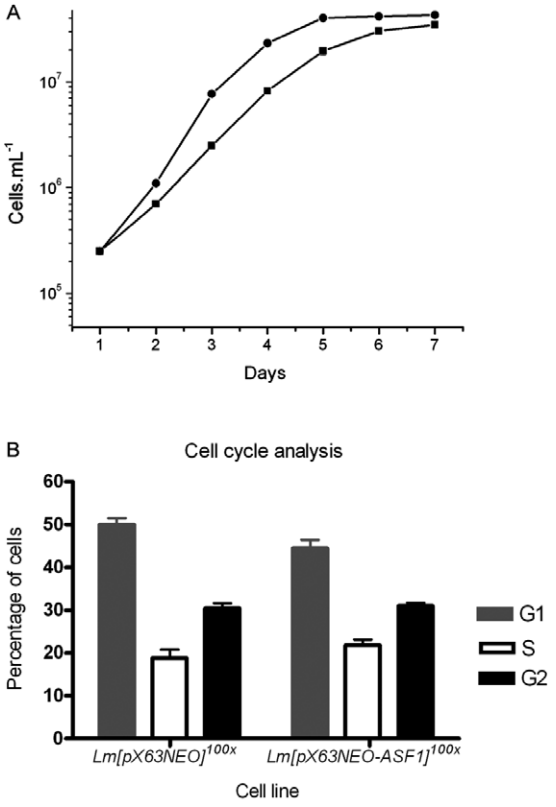

Fig. 3: growth and cell cycle of *LmASF1* overexpressor. A: growth curves of *Leishmania major* anti-silencing factor 1 (ASF1) overexpressor Lm*[pX63NEO-ASF1]*^{100x} (■) and control transfectant Lm*[pX63NEO]*^{100x} (●). Each point in the curves shown represents the mean and standard error from triplicates obtained from one representative experiment; B: FACS analysis of *L. major* ASF1 overexpressor (Lm*[pX63NEO-ASF1]*^{100x} and control line (Lm*[pX63NEO]*^{100x}). Cells were rescued on early logarithmic phase of culture and stained with pI. The bars represent the averages of at least five independent experiments. The error bars indicate the standard deviation. The statistic test used was two-way analysis of variance (GraphPad Prism 4.00) and the variation between the cell lines was not significant. Numbers shown in superscript mode refer to the applied drug pressure (times the LD_{50} of G418). Gray, white and black bars correspond to the cell cycle phases G1, S and G2, respectively.

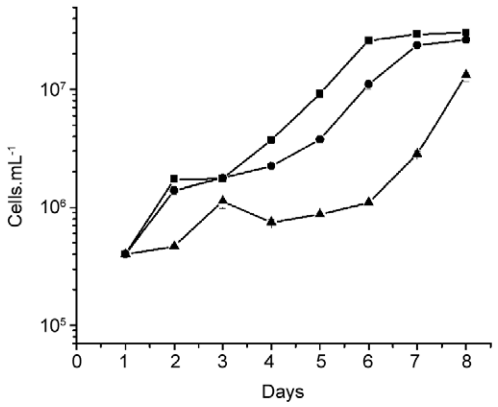

Fig. 4: growth impairment of *Leishmania* anti-silencing factor 1 (ASF1) overexpressors under methyl methane sulfonate (MMS) treatment. *Leishmania major* parental line (LmCC-1) (■), ASF1 overexpressors Lm*[pX63NEO-ASF1]*^{10x} (●) and Lm*[pX63NEO-ASF1]*^{50x} (▲) were maintained in the presence of MMS (0.005%) and monitored for cell growth for eight days. Numbers shown in superscript mode refer to the drug pressure used (times the LD_{50} of G418). Each point in the curves shown represents the mean and standard error from triplicates obtained from one representative experiment.

We also evaluated the overall protein profile modifications triggered by the overexpression of ASF1 in the *L. major* ASF1 mutant. From three independent cultures of each cell line, protein was extracted and fractionated by two-dimensional gel electrophoresis and subsequently identified by mass spectrometry (Supplementary data). The number of identified spots in the 2D gel triplicates varied between 638-672 for the Lm*[pX63NEO]* and 725-990 for Lm*[pX63NEO-ASF1]*. We detected 19 proteins that were differentially expressed. Nine proteins were upregulated in the mutant and 10 were downregulated (Table II). Noteworthy, when we grouped the differentially expressed genes by Gene Ontology website terms (GO) (geneontology.org) we observed that six of them were either classified as heat-shock (HS), HS-related proteins or stress induced proteins (LmjF11.0350, LmjF26.1240, LmjF30.2460, Lmj28.2770, LmjF36.2030, LmjF08.1110), one protein is involved in proteolytic processes (LmjF33.2770), two have been identified as putative nucleosome assembly proteins (coded by genes LmjF29.2340 and LmjF19.0440) and three are involved in oxidoreductase activity (LmjF23.0040, LmjF23.0360, LmjF07.0430) (Table II). The other seven proteins identified were classified in diverse groups of GO categories (Table II).

ASF1 mutant is oversensitive to methyl methane sulfonate (MMS) - To verify whether ASF1 overexpression modifies the response to DNA damaging agents, we evaluated the parasites resistance to an alkylating agent. Parasites were submitted to sub-lethal concentration of MMS (0.005%) and promastigotes multiplication rates were estimated. There was a visible difference between the growth rate of parental cells and ASF1 overexpressor in the presence of MMS. In the *LmASF1* overexpressor the lag phase was extended for five days and only on the sixth day of culture it reached the logarithmic phase of growth, which in the parental line occurred on the third day (Fig. 4).

DISCUSSION

The eukaryotic ASF1 protein is highly conserved throughout evolution (Daganzo et al. 2003, Mousson et al. 2005, Tamburini et al. 2005). Homologues of the *ScASF1* have been found in the genomes of trypanosomatids (tritrypdb.org) and the involvement of the *T. brucei* homologue, TbASF1a, in the control of spindle assembly and S-phase progression has been shown (Li et al. 2007). Nevertheless, no functional studies have been reported with *Leishmania* ASF1 homologues. This is the first study that presents an initial characterization of *L. major* ASF1.

Leishmania ASF1 amino acid sequence shows a high degree (~40%) of identity with homologues of a wide

variety of organisms, from yeast to man (Fig. 1) (ncbi.nlm.nih.gov/). In fact, the trypanosomatid's ASF1 corresponds to the 155-conserved amino-terminal sequence of budding yeast ASF1. Interestingly, we observed conservation among residues V45, V92, L96 and Y112 (Fig. 1B), which are the ones flanking the hydrophobic groove, a potential binding site for histones and other interacting proteins (Daganzo et al. 2003, Mousson et al. 2005). The central residue in this groove is located at position 94. Whereas in yeast this residue is a valine in *Drosophila* and in trypanosomatids it is an isoleucine. The hydrophobicity of the region is maintained by polar and charged residues such as D54 and R108, which are conserved in trypanosomatids. We observed that in *LmASF1* all substitutions of residues that occurred in this groove and surrounding regions are conserved (Fig. 1). This region is crucial for the establishment of electrostatic interactions between the chaperone and the histone core (Mous-

TABLE I

Transcript differential expression of 42 genes by real-time polymerase chain reaction

ID	Product	Distance of telomere (Kb)	Relative expression Lm*[pX63NEO-ASF1]*/ Lm*[pX63NEO]*
LmjF01.0010	Hypothetical protein conserved	3.7	-
LmjF01.0020	Hypothetical protein unknown function	5.8	-
LmjF01.0810	Calcium/potassium channel, putative	24.75	-
LmjF01.0820[a]	Potassium channel subunit-like protein	16.6	2.3x
LmjF02.0010	Phosphoglycan beta 1,3 galactosyltransferase 3	6.87	-
LmjF02.0180	Phosphoglycan beta 1,2 arabidosyltransferase	92.8	-
LmjF02.0190	Phosphoglycan beta 1,3 galactosyltransferase like-protein	97.1	-
LmjF03.0080	Glycerol 3 phosphate acyltransferase	18.8	-
LmjF04.0010	Calcium translocating P-type ATPase organelle-type calcium ATPase	4.6	-
LmjF04.0020[a]	Pteridine transporter (truncated) putative	9.8	1.6x
LmjF04.0030	Camp-specific phosphodiesterase, putative	12.7	-
LmjF04.0060	DNA topoisomerase type IB small subunit	27.7	-
LmjF04.1160	Fructose 1,b biphosphatase, cytosolic putative	43.9	-
LmjF04.1190	tRNA nucleotidyltransferase putative	18.3	-
LmjF04.1220	Hypothetical protein conserved	6.7	-
LmjF08.0080	ATP-dependent DEAD/H RNA helicase putative	21.0	-
LmjF09.0070	RNA helicase putative	17.2	-
LmjF11.0010	Hypothetical protein conserved	1.9	-
LmjF11.0020	Hypothetical protein conserved	2.9	-
LmjF11.0030	Hypothetical protein conserved	4.6	-
LmjF11.1360	Hypothetical protein conserved	10.6	-
LmjF11.1370	Hypothetical protein conserved	7.1	-
LmjF11.1380	Hypothetical protein conserved	4.0	-
LmjF18.0010	Hypothetical protein conserved	4.7	-
LmjF18.0020	Diphosphomevalonate decarboxylase, putative	7.9	-
LmjF18.0030	Hypothetical protein conserved	9.7	-
LmjF18.1660	Gamma-glutamylcysteine synthetase	10.0	-
LmjF18.1670	Hypothetical protein, unknown function	4.0	-
LmjF20.0020	Hypothetical protein conserved	7.5	-
LmjF20.0030	Hypothetical protein conserved	9.4	-
LmjF20.0040	Hypothetical protein conserved	13.2	-
LmjF20.0050[a]	Anti-silencing protein 1-like protein	15.1	90.5x
LmjF20.0060	Hypothetical protein conserved	19.6	-
LmjF20.0070	Hypothetical protein conserved	21.3	-
LmjF20.0080[a]	Hypothetical protein conserved	24.9	0.57x
LmjF20.0090[a]	Hypothetical protein conserved	30.3	0.62x
LmjF20.160	Hypothetical protein conserved	3.2	-
LmjF22.1670	Hypothetical protein conserved	32.8	-
LmjF23.0290	Hypothetical protein conserved	108.2	-
LmjF23.0300	Tryptophanyl-tRNA synthase, putative	110.1	-
LmjF23.0310	Hypothetical protein conserved	112.8	-
LmjF23.0320[a]	Hypothetical protein conserved	118.2	0.48x
LmjF23.0330	Hypothetical protein conserved	120.0	-

a: genes affected by anti-silencing protein 1 (ASF1) overexpression; ID: gene identification as annotated in the *Leishmania major* genome.

son et al. 2005). It has been shown (Daganzo et al. 2003) that this core domain is sufficient to confer resistance to DNA damage agents and to guarantee interaction of ASF1 with its partners, H3/H4 tetramer and Rad53. This conservation of the N-terminal region of *LmASF1* indicates that the hydrophobicity of this concave groove and its surrounding regions is maintained in *L. major,* which suggests that interaction of ASF1 with these partners is maintained in *Leishmania*.

We generated *Leishmania* parasites that produces higher amounts of ASF1 transcripts (90.5 times higher) and protein (at least 3 times higher) when compared to the control. The difference between ASF1 transcript and protein levels in the mutant is remarkable. This finding led us to speculate that this protein at high levels is deleterious to the parasite and that degradation of the excess of protein is necessary for the maintenance of cell homeostasis. Alternatively, the mRNA produced is degraded before being translated. These hypotheses are supported by other observations made in the course of this study. One of them is the fact that the ASF-overexpressing parasite presented a delayed growth, with a doubling time of 8.67 h compared to 6.84 h observed in the control line (Fig. 3A). Another possibility to explain the growth rate decrease is that cells have a delay in the S phase, a finding compatible with the fact that Asf1A is a substrate for Tousled-like kinases implicated in DNA replication control in *T. brucei* (Li et al. 2007). Nevertheless, we have not observed more than a subtle apparent increase on S-phase non synchronized cells, which was not confirmed in experimental triplicates (Fig. 3B).

ASF1 was originally identified as a disrupter of silencing of specific loci in *S. cerevisiae* (Le et al. 1997, Singer et al. 1998). In yeast, position-dependent gene silencing, including telomere silencing, involves many factors responsible for chromatin/nucleosome assembly, for instance ScAsf1p (Sharp et al. 2001). The fact that telomere proximal domains are less accessible to the transcriptional machinery, as compared to other chromosome regions, offers protection to these regions (Gottschling 1992). In several organisms, including *T. brucei,* this is achieved by repression of nearby genes (Levis et al. 1985, Gottschling et al. 1990, Zomerdijk et al. 1991, Horn & Cross 1995), with the control of chromatin packaging being essential for the strict control of the variant surface glycoproteins expression in these parasites (Rudenko 2010). To search for a potential role for *LmASF1* over telomere-proximal genes transcriptional control, we quantified the transcript level of 42 genes of *Leishmania,* mostly from the telomere proximal domains. We found that the augmented levels of ASF1 did not change the expression levels of any of the telomere-proximal genes analyzed (Table I), suggesting that differently than *S. cerevisiae*'s ASF1, the parasitic homologue is not involved in the expression control of telomeric genes. Nevertheless, we cannot rule out that a different factor plays this function in *Leishmania*.

Through proteomic analysis we investigated the potential changes that overexpression of ASF1 induced in gene expression. Interestingly, we observed changed levels of two proteins related to proteolytic activity (Table II). These proteins are the ubiquitin conjugating enzyme (Lmj33.2770) and a metalloprotease (LmjF05.0960). In addition, there is a third protein that might be also involved in proteolysis; it is classified as an ATPase beta subunit protein (LmjF25.1170) that might be a member of the AAA ATPase family, which participates in a variety of cellular processes, including the shuttle of a subset of ubiquitinated substrates to the proteasome for degradation (Ye 2006). An indication that the ASF1 mutant was under stress comes from the overexpression of genes involved in physiological stress processes (LmjF11.0350, LmjF26.1240, LmjF30.2460, Lmj28.2770, LmjF36.2030, LmjF08.1110) (Table II, Supplementary data). Furthermore, the detection of altered levels of three proteins involved in the oxidoreductase activity (LmjF23.0040, LmjF23.0360, LmjF07.0430) suggests that cell redox metabolism is affected in the mutant.

Because ASF1 has a wide spectrum of functions related to chromatin metabolism, control of ASF1 levels must be critical for cell viability. ASF1 post-translational modifications were shown to be relevant for the control of its own cellular levels. It has been shown that phosphorylation of human and *Drosophila* ASF1 by tousled-like kinases (TLK) positively regulates protein levels, because it hampers the proteasome-dependent degradation of ASF1 (Pilyugin et al. 2009). In *T. brucei*, both ASF1 isoforms interact and are phosphorylated by TLK1 (Li et al. 2007). Currently, we have no information on the occurrence of post-translational modifications of *LmASF1*, nor whether or not it interacts with the *L. major* TLK. Assuming that the post-translational process happens similarly to that in *T. brucei*, we speculate that the endogenous LmTLK is unable to phosphorylate the excess of ASF1 produced by the mutant and that the unstable ASF1 is ubiquinated and shuttled to the proteasome to recover the organism homeostasis.

Finally, consistent with the *LmASF1* role in the control of chromatin dynamics, which interacts with other CAFs, we found in the comparative proteomic analysis (Table II, Supplementary data) that two of the proteins found in higher levels in the ASF1 mutant are involved in chromatin remodelling (LmjF29.2340 and LmjF19.0440). Interaction of these proteins with ASF1 must be further investigated.

It has been shown that in other organisms ASF1 contribution to DNA repair is also linked to its effect on assembly/disassembly of chromatin, which is similar to what has been shown for its DNA replication or control of transcription (Mello et al. 2002, Gontijo et al. 2003, Green & Almouzni 2003, Canfield et al. 2009). The remarkable decrease in multiplication rates of ASF1 mutant in the presence of MMS indicates that this *LmASF1* isoform modifies the response to DNA damage. It is possible that high levels of ASF1 affect the chromatin structure, making the overexpressor's DNA more susceptible to the genotoxic effect of MMS. Alternatively, the delayed growth rate of the ASF1 overexpressor may be the result of the parasite attempt to maintain cellular homeostasis combined to the deleterious effects of the genotoxic agent.

TABLE II

Identification of proteins differentially expressed in Lm*[pX63NEO-ASF1]* as compared to Lm*[pX63NEO]*

MS ID	Differentially expressed proteins spot identification	Accession	Gene Ontology (GO)		1st D (pI) theo/exp[a]	2nd D (MW.kDa) theo/exp[b]	Mascot score[c]	Normalized volume[d]	
			GO ID	GO term name				Lm *[pXN63NEO]*	Lm *[pX63NEO-ASF1]*[e]
25	No identified	-	-	-	?/6.4	?/16	-	0.133	-
28	MaoC family dehydratase-like protein from *Leishmania major*	LmjF07.0430	0008152	Metabolic process	5.1/5.1	16/17	137	0.055	0.109 (+1.55)
44	Hypothetical protein from *L. major*	LmjF29.2340	0006334	Nucleosome assembly	4.6/4.5	21/20	102	0.113	0.199 (+1.50)
54	Tryparedoxin peroxidase from *L. major*	LmjF23.0040	0045454	Oxidoreductase activity	4.4/5.5	25/22	194	1.251	0.865 (-1.25)
73	Ubiquitin-conjugating enzyme from *L. major*	LmjF33.2770	0006508	Protein modification process/proteolysis	7.0/6.3	25/24	119	-	0.254
133	Chaperonin Hsp60, mitochondrial precursor from *L. major*	LmjF36.2030	0006457	Cellular protein metabolic process/protein folding	5.3/5.3	59/33	286	0.344	0.198 (-1.62)
175	NADP-dependent alcohol dehydrogenase from *L. major*	LmjF23.0360	0008152	Oxidoreductase activity	5.8/6.3	38/38	201	0.376	0.845 (+1.83)
194	Heat shock 70-related protein 1, mitochondrial from *L. major*	LmjF30.2460	0006457	Protein folding/unfolded protein binding	5.5/6.4	68/40	171	0.155	0.306 (+1.67)
222	Nucleosome assembly protein from *L. major*	LmjF19.0440	0006334	Nucleosome assembly	4.6/4.7	39.7/45	89	0.141	0.238 (+1.54)
252	ATPase beta subunit from *L. major*	LmjF25.1170	0015986	ATP synthesis coupled proton transport	5.1/5.1	56/50	157	0.040	0.079 (+1.56)
266	Dihydrolipoamide acetyltransferase precursor from *L. major*	LmjF36.2660	0008152	Acyltransferase activity/ protein binding	7.0/6.4	49/53	87	0.060	0.134 (+1.57)
300	Protein antigen LmSTI1 from *L. major*	LmjF08.1110	none	None	5.9/6.2	62/61	87	0.310	0.137 (-1.89)
356	Heat shock protein 70-related protein from *L. major*	LmjF26.1240	0005524	ATP binding	5.0/4.8	71/39	91	-	0.069
405	Alpha tubulin from *Leishmania infantum*	LmjF13.0280	0007017	Microtubule-based movement	6.8/5.5	36/21	100	0.075	-
407	Peroxidoxin; tryparedoxin peroxidase from *Leishmania*	LmjF23.0040	0045454	Oxidoreductase activity	6.4/5.3	25/22	83	0.136	-
411	"14-3-3 protein" from *L. major*	LmjF11.0350	0019904	Protein domain Specific binding	5.3/5.4	29-30/28	85	0.122	-
414	Hypothetical protein from *L. major*	LmjF25.2020	none	None	5.5/5.7	32/29	152	0.089	-

↳

MS ID	Differentially expressed proteins spot identification	Accession	Gene Ontology (GO) GO ID	GO term name	1st D (pI) theo/exp[a]	2nd D (MW.kDa) theo/exp[b]	Mascot score[c]	Normalized volume[d] Lm *[pXN63NEO]*	Lm *[pX63NEO-ASF1]*[e]
426	Activated protein kinase c receptor (LACK) from *L. major*	LmjF28.2740	0032465	Regulation of cytokinesis/ protein binding	6.0/6.4	34/36	234	0.077	-
478	Heat-shock protein 70 from *L. major*	LmjF28.2770	0005524	ATP binding	5.3/5.5	71-75/63	84	0.084	-

a: the theoretical (theo) pI value over the experimental (exp) pI found for each given protein; *b*: the theo molecular weight (MW) for each given protein over the encountered MW values in the experiment; *c*: quantification of the identification probability of the fragment match. A definition and interpretation of the meaning of the Mascot score can be found at the website matrixscience.com. We have included only statistically significant Mascot score results ($p < 0.05$). Protein spots with statistically non significant Mascot scores remained unidentified; *d*: it corresponds to the amount of protein detected in each spot, in each strain. It is expressed as volume of the spot relative to the total volume of protein loaded in the gel. Therefore, the estimation of the difference between the volume of the spot in the compared strains is precisely expressed and allows to estimate the intensity of each signal comparatively in a given gel. When the spot was not detected in one strain a (-) signal indicates it; *e*: positive numbers (+) represent the fold difference of the upregulated proteins in the mutant strain and negative numbers (-) represent those proteins that are downregulated in the mutant, comparatively to the control strain; ASF1: anti-silencing protein 1; NADP: nicotinamide adenine dinucleotide phosphate.

Overall, our results indicate that the *Leishmania* ASF1 is not involved in controlling the expression of telomeric genes. On the other hand, altered levels of nucleosome assembly proteins (putative partners of ASF1) and the delayed growth rate of ASF1 overexpressor when submitted to a clastogenic agent indicate that ASF1 is involved in chromatin assembly/disassembly. Changed levels of proteins potentially involved in physiological stress conditions and in proteolytic processes, combined with the observed impaired growth rates, indicate that overexpression of ASF1 is deleterious to the parasite. Chromatin assembly dynamics is relevant for controlling transcription in trypanosomatids (Green & Almouzni 2003, Figueiredo et al. 2009) and is central for the DNA repair machinery. Investigating the roles and interactions of *Leishmania's* ASF1 is a route to improve understanding of how this important biological process may occur in trypanosomatids.

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