Expression of a Novel *Leishmania* Gene Encoding a Histone H1-Like Protein in *Leishmania major* Modulates Parasite Infectivity In Vitro

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We describe identification and characterization of a novel two-copy gene of the parasitic protozoan *Leishmania* that encodes a nuclear protein designated LNP18. This protein is highly conserved in the genus *Leishmania*, and it is developmentally regulated. It is an alanine- and lysine-rich protein with potential bipartite nuclear targeting sequence sites. LNP18 shows sequence similarity to H1 histones of trypanosomatids and of higher eukaryotes and in particular with histone H1 of *Leishmania major*. The nuclear localization of LNP18 was determined by indirect immunofluorescence and Western blot analysis of isolated nuclei by using antibodies raised against the recombinant protein as probes. The antibodies recognized predominantly a 18-kDa band or a 18-kDa–16-kDa doublet. Photochemical cross-linking of intact parasites followed by Western blot analysis provided evidence that LNP18 is indeed a DNA-binding protein. Generation of transfectants overexpressing LNP18 allowed us to determine the role of this protein in *Leishmania* infection of macrophages in vitro. These studies revealed that transfectants overexpressing LNP18 are significantly less infective than transfectants with the vector alone and suggested that the level of LNP18 expression modulates *Leishmania* infectivity, as assessed in vitro.

*Leishmania* spp. are obligately intracellular protozoan parasites that are members of the family Trypanosomatidae, and they cause a wide spectrum of human diseases of major health importance, ranging from self-healing cutaneous lesions to severe visceral leishmaniasis or kala-azar with a high fatality rate (13). These parasites have two developmentally distinct stages; the amastigotes are nonmotile forms that survive and replicate within mammalian host macrophages, and the motile flagellated promastigotes survive and replicate inside the insect vector (13).

During the last several years, considerable interest has been focused on an extensive study of the biology of these kinetoplastid protozoans, which are among the most primitive eukaryotes (48). *Leishmania* has assumed importance in molecular biology by virtue of the unusual nature of its gene organization and expression (39). The unusual features include the presence of multiple copies of the same gene organized in tandem arrays or adjacent genes encoding different proteins, which are often transcribed as large polycistronic precursors of mature mRNAs (32, 37), RNA editing, and transsplicing (38, 47). *Leishmania* molecular genetic studies have provided new insights into the mechanisms of gene expression (reviewed in reference 38). However, very little is known about gene regulation in these protozoans.

In all eukaryotic cells, the DNA is highly compacted due to its association with histone proteins (33). The basic structural unit of chromatin is the nucleosome, which comprises DNA wrapped tightly around an octameric-histone core having a tripartite organization consisting of a central (H3-H4)2 tetramer flanked by two H2A-H2B dimers (1). Histones, despite their low sequence homology, have a common motif, the histone fold (1, 2), which is considered to be a general protein dimerization motif. Most of the proteins classified in the histone fold superfamily are involved in protein–protein and/or protein–DNA interactions (2, 6). This motif is found in a wide variety of transcriptional activators resembling histones (11), such as the archaeal DNA-binding proteins HMi, HMt, and HMv (54), the CCAAT-specific transcription factor CBF (60), and the TAFII42 and TAFII62 subunits of the TFIID transcriptional complex of *Drosophila* (30). The linker histones H1 and H5 are essential for the organization of nucleosomes into a higher-order structure (43, 55). Like the relationship of core histones, a relationship between linker histones and transcription factors has also been suggested (16, 35).

There is great interest in histone genes in trypanosomatids because in these organisms chromatin is not condensed into chromosomes during cell division but remains decondensed as fine fibers. However, the DNA is associated, probably weakly, with all classes of histones and is packed into nucleosomes (4). Characterization and systematic studies of the *Trypanosoma* genes coding for histones (3, 8, 25, 42), as well as the *Leishmania* genes coding for histones (21, 26, 52, 53), have shown that the sequence similarity with the genes coding for histones in higher eukaryotes is low (reviewed in reference 24). In particular, the H1 histones of trypanosomatids have significantly lower molecular masses than the H1 histones of higher eukaryotes, and there is only 43.6% similarity between the *Trypanosoma cruzi* and human H1 sequences. Moreover, in contrast to the histone genes of higher eukaryotes, trypanosomatid histone genes are located on different chromosomes, and their transcripts are polyadenylated. Although histone genes and their expression in trypanosomatids have been studied extensively, little is known about the regulation of their expression (reviewed in reference 24).

In this paper we describe molecular cloning and character-
ization of a novel histone H1-like Leishmania nuclear DNA-binding protein, LNP18, and present evidence that LNP18 plays a role in Leishmania infectivity.

MATERIALS AND METHODS

In vitro culture of Leishmania. Promastigotes were grown at 26°C in Dulbecco modified Eagle medium (Gibco) containing 10% heat-inactivated fetal calf serum. The following strains were used in this study and in previous studies: Leishmania major LV39 and Leishmania infantum HOM-Gr78L4, isolated in Greece (57, 58), and Leishmania amazonensis LV78, kindly provided by K.-P. Chang. Amastigotes were isolated from lesions (at least 2 months old) of BALB/c mice infected with 1 × 10⁹ stationary-phase L. major promastigotes by using established protocols (28).

Screening of a cDNA library and DNA sequence analysis. An L. major cDNA library was constructed in the lambda Uni-ZAP XR vector as described in the technical manual provided by Stratagene Inc. (La Jolla, Calif.). Poly(A)+ mRNA from L. major promastigotes was used to synthesize cDNAs. A cDNA clone that was recognized by affinity-purified antibodies raised against the purified Leishmania transferrin receptor molecule (59) was isolated and sequenced. Leishmania cDNAs in the Uni-ZAP XR vector were subsequently plated on Escherichia coli X1-Blue plates, and the phagemids were excised by the methods described in the manufacturer’s protocol manual (Stratagene Inc.). Subsequently, plasmid DNA from the phagemids was isolated and sequenced by using standard procedures (45).

Southern and Northern blot hybridization analyses. DNAs were isolated from promastigotes, digested to completion (3 h at 37°C) with endonuclease Xbal (5 U/μg of DNA), electrophoresed on 0.8% agarose containing 45 mM Tris, 45 mM boric acid, and 1 mM EDTA (pH 8), and transferred to nylon membranes; the agarose was stained with ethidium bromide. Plasmid DNA in Leishmania cells was isolated by the alkaline lysis method (51). Total cellular RNA was extracted from Leishmania promastigotes by the hot acid phenol method as modified by Brown and Kalatos (10). For Northern blot analysis, total parasite RNA was electrophoresed through a 1.2% (wt/vol) agarose gel containing formaldehyde and was subsequently transferred to blotting membranes (Zetaprobe; Bio-Rad). Hybridization and washing for either DNA or RNA analysis were carried out as previously described (51). Filters were hybridized overnight at 65°C with 32P-labeled cDNA probes, labeled by the random priming method (22), in hybridization solution containing 1% crystalline grade bovine serum albumin, 1 mM Na2EDTA (pH 7.2), and 7% (wt/vol) sodium dodecyl sulfate (SDS). The filters were washed as described by Church and Gilbert (14). The membranes were autoradiographed on X-Omat AR film (Kodak) at –80°C with two intensifying screens. For rebinding of a membrane, the probe was eluted by two washes with 2 mM Tris-EDTA (pH 8.2)–0.1% (wt/vol) SDS for 15 min at 95°C.

The amounts of RNA loaded on filters were estimated by ethidium bromide staining of agarose gels loaded with equivalent amounts of the samples, as well as by staining the nitrocellulose filters with methylene blue in order to evaluate the amounts of transferred rRNAs.

Probes. The following probes were used in this study: pBS10/Rh1, containing a 2.2-kb EcoRI fragment from an L. major gp63 cDNA clone (12), kindly provided by L. Button and R. McMaster (Medical Genetics, University of British Columbia, Vancouver, Canada); and T11, containing a 2-kb PstI fragment from an L. amazonensis β-tubulin cDNA clone, T11 (23), kindly provided by K.-P. Chang (Department of Microbiology and Immunology, University of Health Sciences, The Chicago Medical School, North Chicago, Ill.).

Production of antibodies. Anti-LNP18 antibodies were obtained from rabbits immunized with recombinant LNP18 (rLNP18). rLNP18 was produced by using the pRSET-E Echo cloning system (Invitrogen, Groningen, The Netherlands). In the Echo cloning system a one-step cloning strategy is utilized for direct insertion of a PCR product into an appropriate plasmid vector, and the classical cloning procedures are not required. The fusion protein was transformed into Top10 cells, and positive clones were analyzed. To confirm the fusion junctions, isolated plasmid DNA was analyzed by restriction analysis and sequencing. After overexpression in E. coli, rLNP18 was purified by affinity chromatography on Nα⁺-nitrotriacetate resin columns under denaturing conditions by following the supplier’s instructions (Qiagen). Two New Zealand White rabbits were immunized subcutaneously three times every 2 weeks with 50 μg of rLNP18. Affinity-purified anti-LNP18 antibodies were isolated by low-pH elution from immunoblots of purified rLNP18 as previously described (59).

Gel electrophoresis and immunoblotting. SDS-polyacrylamide gel electrophoresis (PAGE) was performed on 12.5% polyacrylamide gels by the method of Laemmli (34). The separated proteins were then transferred to nitrocellulose, and a Western blot analysis (56) was carried out essentially as previously described (50). The filters were blocked overnight at 4°C with 5% powdered nonfat milk in Tris-buffered saline. After incubation with horseradish peroxidase-conjugated secondary antibodies, bands were detected with X-ray film with enhanced sensitivity (0.03% [wt/vol] DAB and 0.03% [wt/vol] NiCl2 in Tris-buffered saline).

Nucleus preparation. Nuclei were prepared as described by Hinterberger et al. (29). Briefly, stationary-phase promastigotes (10⁶ cells) were washed and lysed in 6 ml of NB buffer containing 10 mM HEPES, 10 mM MgCl₂, 2 mM dithiothreitol, 0.5% (wt/vol) BSA, 0.05% (vol/vol) Triton X-100 in a Dounce homogenizer. The lysate was centrifuged (20 min, 1,900 × g), and the resulting pellet was washed in NB buffer and centrifuged at 100,000 × g for 90 min at 4°C over a 2 M sucrose cushion in NB buffer containing 0.5% (wt/wt) Triton X-100. The nuclear pellet was subsequently washed and resuspended in 50 mM HEPES–5 mM MgCl₂–2 mM dithiothreitol–1 mM EDTA containing 40% (vol/vol) glycerol.

Immunofluorescence staining. Promastigotes (10⁶ cells/ml) were washed in phosphate-buffered saline (PBS) and fixed on glass microscope slides as previously described (49). Antibodies were diluted in PBS containing 0.3% (wt/vol) bovine serum albumin. The primary antibody was then applied overnight at 4°C, and the fluorescence-conjugated secondary antibody was applied for 30 min at room temperature. Coverslips were mounted on the glass slides in 90% glycerol in PBS, and the preparations were viewed either with a Zeiss Axioshot photomicroscope or with a Leica laser scanning confocal microscope.

In vitro UV cross-linking of nucleic acids to proteins. For in vitro cross-linking of DNA to proteins, we used the approach used by Pelle and Murphy (41). Briefly, isolated nuclei from approximately 8 × 10⁹ L. major promastigotes were homogenized on ice in 200 μl of homogenization buffer (25 mM Tris-Cl, 1 mM EDTA; pH 8) in a Dounce homogenizer. The lysate was centrifuged (20 min, 1,900 × g), and the resulting nuclear extract was subsequently digested with 5 U of Smal per μl. Ten microliters of the nuclear extract in 400 μl of 25 mM HEPES–120 mM NaCl–6 mM KCl–2 mM MgCl₂ (pH 7.4) was incubated at room temperature for 20 min before UV irradiation. Irradiation was carried out with a 254-nm UV light for 20 min at 4°C. After UV treatment, 2 volumes of cold ethanol was added, and the precipitate was pelleted by centrifugation. The resulting pellet was dissolved in SDS-PAGE sample buffer, boiled for 5 min, and then separated by SDS-PAGE and analyzed by Western blotting by using the anti-LNP18 antibodies as probes. For the control we used samples treated with 100 U of DNase I per ml before or after UV cross-linking and samples prepared by a procedure from which the irradiation step was omitted.

Plasmid constructs. The pX63-HYG leishmanial expression vector (17) was kindly provided by S. Beverley. The cDNA of LNP18 was end filled with the Klenow fragment of DNA polymerase I (New England Biolabs, Hertfordshire, United Kingdom) and cloned into the Smal restriction site of the pX63-HYG polylinker.

Transfection of Leishmania. L. major LV39 stationary-phase parasites were transfected with approximately 10 μg of pX63-HYG carrying the LNP18 gene and with pX63-HYG alone as a control. Cells were subjected to electroporation with a Gene Pulser II (Bio-Rad) by using established protocols. After 24 h of recovery in hygromycin B-free medium 199, resistant cells were selected in the presence of increasing concentrations of hygromycin B (up to 500 μg/ml).

RT-PCR. Total RNA was extracted from transfected LV39 promastigotes by using an RNasey kit for RNA isolation (Qiagen) according to the manufacturer’s instructions. cDNA synthesis and a PCR were performed in an one-step reaction by using the Titan one-tube reverse transcription (RT)-PCR system (Roche). For PCR the following LP18F and β-actin-derived oligonucleotides were used: LP18F (5′-AGGGTCCTGCTACCGCAGGAC-3′) and LP18R (5′-ACTGCTGAGCANGTAGACAC-3′); and β-actinF (5′-GACTCCCTATGGGTTGGCTGAC-3′) and β-actinR (5′-GGGGAGGACATGACCCCTGTAGAT-3′). Each PCR was performed in a 50-μl reaction mixture containing 10 μl of RT-PCR buffer, 25 pmol of each primer, 5 μl of a solution containing each deoxynucleoside triphosphate at a concentration of 2.5 mM (Promega), and 1 μl of enzyme mixture. In addition, 0.5 μl of an RNase inhibitor (RNasin; 40 U/μl Promega) was added. The amplification cycle (denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C) was repeated 30 times, and this was followed by a 7-min final extension step.

Infection of macrophages. Macrophages of the J774 line (American Type Culture Collection, Manassas, Va.) were plated in 96-well flat-bottom microcul-
FIG. 1. Nucleotide sequence of L. major LNP18 cDNA. The deduced amino acid sequence is indicated below the DNA sequence. The domain that contains the eight potential sites of bipartite nuclear sequences (bipartite nuclear localization signals) is underlined. The amino acid sequence is indicated by the single-letter amino acid code.

Fluorescence-activated cell sorting analysis was performed for infected and noninfected macrophages as described by Di Giorgio et al. (18) by using antibodies generated against LV39 lesion-derived amastigotes. Cells were incubated with antibodies in permeabilization buffer containing 4% v/v paraformaldehyde, 2% v/v glutaraldehyde, and 0.1% w/v sodium azide in PBS (pH 7.4) for 30 min (62). Schneider’s medium supplemented with 10% fetal calf serum was added, and the released parasites were incubated at 26°C for 48 h. Cultures were subsequently pulsed with 1 &mu;Ci of &superscript;3H&thinspace;thymidine per well, and the radioactivity incorporated was counted with a FacSort analytical cytometer (Becton Dickinson). The nucleotide sequence reported in GenBank, accession number X18304, has been deposited with the EMBL, GenBank and DDBJ databases under accession number M18935.

RESULTS

Cloning and sequencing of the LNP18 cDNA. In an effort to clone and characterize the Leishmania transferrin receptor gene, a molecule that was first identified in our laboratory (59), a cDNA clone encoding a novel protein, LNP18, was accidentally identified and subsequently sequenced. Figure 1 shows the complete nucleotide sequence of the 0.690-kb cDNA insert and the deduced amino acid sequence. The deduced amino acid sequence predicts a basic protein (pl 12.2) containing 98 amino acids and having a predicted molecular mass of 9.9 kDa.

The LNP18 predicted amino acid sequence was compared with the sequences of proteins listed in SBASE by advanced BLAST. This search revealed that LNP18 belongs to the histone H1-histone H5 family. Interestingly, LNP18 shows significant similarity with H1 histones of members of the Trypanosomatidae. Sequence alignments for LNP18 and the H1 histones of T. cruzi and other Leishmania species, obtained by using CLUSTALW, are shown in Fig. 2A. It should be noted that H1 histones in eukaryotes are less conserved than the core histones and exhibit a high degree of variability. Moreover, H1 histones in members of the Trypanosomatidae have much lower molecular masses than their eukaryotic counterparts and exhibit exclusive motifs characteristic of the C-terminal domain of eukaryotic H1 histones (reviewed in reference 24). Galanti et al. found 43.6% similarity between T. cruzi H1 and the C-terminal region of human H1 (24). Furthermore, the L. major (21) and T. cruzi H1 histones (24) exhibit 61.79% similarity (data not shown). The sequence of LNP18 was compared with that of L. major histone H1. This comparison (Fig. 2B) showed that 61% of the residues are either identical (56%) or well conserved (5%). Thus, LNP18 is a histone H1-like protein.

Analysis of the sites and signatures of the LNP18 amino acid sequence revealed that it is an alanine- and lysine-rich protein with eight potential sites of bipartite nuclear localization signals (Fig. 1). Bipartite nuclear localization signals are known to be necessary for nuclear targeting (5, 19, 44, 63). Interestingly, the same motif is present in almost one-half the nuclear proteins in the Swiss protein database but in less than 5% of the nonnuclear proteins, and many of these proteins are secreted or targeted to other organelles. An example of a molecule having a relatively low molecular mass (143 residues) that has a functional nuclear localization signal-like motif is human gamma interferon (63). A hydrophobicity analysis performed by using PCGENE suggests that LNP18 is a globular protein (data not shown).

Genomic organization and expression of the LNP18 gene: Southern and Northern analyses. Southern blot analysis was performed to determine the copy number of the LNP18 gene by using genomic DNAs from L. major and L. infantum promastigotes. The DNAs were digested with the following restriction enzymes, separately or in combination: EcoRI, HindIII, and BglI, which have no internal sites within the cDNA; and EcoR109I and AvaI, which have one internal site. As shown in Fig. 3A, digestion with enzymes having no internal sites within either the L. major cDNA (lanes 1 to 3) or the L. infantum genomic DNA (lanes 8 and 9) resulted in two bands when the preparations were hybridized (under highly stringent conditions) with a probe containing the complete LNP18 cDNA. If the physical maps of sw3.0 and sw3.1 and the physical map of LNP18 (Fig. 3B) are taken into account, the possibility of cross-hybridization of the LNP18 cDNA probe with fragments of the two L. major histone H1 genes cannot be excluded. However, it has been reported that when the sw3.0 open reading frame is used as a probe in Southern blots, it does not detect any digests in Leishmania donovani, a species that belongs to the same complex as L. infantum (7). This finding supports the assumption that at least in L. infantum the two fragments detected correspond to digests of the LNP18 locus. When the L. major DNA was digested with AvaI (Fig. 3A, lane 5), three bands, at 1.9, 1.75, and 0.85 kb, were observed; the 0.85-kb band probably corresponds to an sw3.1 fragment of the L. major H1 gene (Fig. 3B, map 1). BglI digestion resulted in two bands, at 2 and 1.8 kb (Fig. 3A, lane 3). However, AvaI-BglI double digestion resulted in a 0.85-kb band (Fig. 3A, lane 3).
that could correspond to the AvaI fragment detected in Fig. 3A, lane 5. The possibility that this fragment could be a digestion product of the sw3 locus that cross-hybridized with the LNP18 probe, due to the high homology of the two genes, cannot be excluded. However, two of the three bands detected in Fig. 3A, lane 4 (the 0.75- and 0.7-kb bands), cannot be attributed to fragments of *L. major* since there are no internal BglII sites in the AvaI fragments of the two H1 gene sequences in the region that shows homology to the LNP18 cDNA probe (Fig. 3B). The latter finding is consistent with the hypothesis that *L. major* LNP18 is a two-copy gene.

Transcription of the gene was confirmed by Northern blot analysis of total RNAs extracted from diverse species of Old World and New World *Leishmania* promastigotes. As shown in Fig. 4A, under highly stringent conditions the probe recognized two transcripts (approximately 0.8 and 0.7 kb). The sizes of these transcripts are consistent with the size of the reported full-length cDNA. These two transcripts may be due to multiple polyadenylation sites or to two closely related genes or copies. Subsequently, we examined the level of expression of LNP18 in the amastigote stage of *L. major* parasites (Fig. 4A, lane 4). Estimation of the amount of RNA transferred onto filters, as described in Materials and Methods, showed that the level of amastigote RNA was at least four times higher than the level of promastigote RNA. On the other hand, the level of the 0.8-kb transcript in *L. major* promastigotes was 15- to 20-fold higher than the corresponding level in amastigotes, as determined by scanning densitometry of a blot that was exposed for a shorter period of time (data not shown). This was evaluated by simultaneously hybridizing the filter with the LNP18 cDNA probe and a 2.2-kb EcoRI fragment from *L. major* gp63 cDNA. gp63 is down-regulated at both the mRNA and protein levels in amastigotes. Interestingly, only one transcript (0.8 kb) was detected in *L. major* amastigotes, but the level was significantly lower (Fig. 4A, lane 4). Most probably the transcript detected was due to cross-hybridization of the LNP18 probe with the histone H1 transcript due to overloading of the gel. Histone H1 is up-regulated in amastigotes compared to promastigotes (21, 40).

The level of expression of LNP18 in amastigotes was also analyzed by RT-PCR. To do this, RNAs were isolated from lesion-derived amastigotes and promastigotes and were amplified by using primers designed from the LNP18 open reading frame. Using this approach, we showed that LNP18 expression was significantly lower in amastigotes than in promastigotes (Fig. 4B, lanes 4 and 2, respectively). Importantly, LNP18 protein was not detected in *L. major* amastigotes (Fig. 5B).

**Identification and localization of native Leishmania LNP18.**

Anti-LNP18 antibodies, generated by using rLNP18, recognized in Western blots of *L. major* lysates an 18-kDa protein or an 18-kDa–16-kDa doublet (Fig. 5A, lane 1). The antibodies recognized the same bands for different *Leishmania* species, indicating that LNP18 is a highly conserved protein (Fig. 5A). The LNP18 antibodies do not seem to cross-react with the closely related H1 protein, since they do not recognize any polypeptides in lesion-derived amastigotes (Fig. 5B lane 1); it has been demonstrated in several studies that expression of H1, at both the mRNA and protein levels, is significantly
higher in lesion-derived amastigotes than in *L. major* promastigotes (21, 40). In order to ensure that the amount of amastigote protein transferred was sufficient to detect LNP18, blots were cohybridized with antibodies against α-tubulin, a protein that is down-regulated in the amastigote stage of *L. major* (15). The fact that the intensities of α-tubulin in the two stages were comparable suggests that the amount of protein loaded in lane 1 (amastigotes) of Fig. 5B was severalfold higher than the amount of protein loaded in lane 2 (promastigotes). Even under these experimental conditions LNP18 was not detected in amastigotes. Therefore, the difference in the level of expression of LNP18 between the two stages is even greater than the difference shown in Fig. 5B.

The molecular mass of purified rLNP18 was also estimated...
to be 18 kDa (data not shown), a value that is significantly higher than the value expected from the deduced amino acid sequence. It should be noted that it is becoming increasingly evident that very basic or acidic proteins may migrate anomalously on SDS-PAGE gels (31). Therefore, the anomalous migration of LNP18 might be due to abnormal binding of SDS to basic amino acids, since LNP18 is a Lys-rich basic protein, which in turn might lead to an abnormal shape of the SDS-protein complex. It should be pointed out that anomalous migration of LNP18 in SDS-PAGE gels is a common characteristic of the latter protein deduced from the cDNA predicts that the protein has 105 amino acid residues. However, specific antibodies recognize a 17-kDa–19-kDa doublet in different L. major strains (40). Like H1 polymorphism, LNP18 size polymorphism may reflect the presence of LNP18 variants possessing common epitopes. Moreover, Western blot analysis of the transfecteds overexpressing LNP18 showed that the bands detected were derived from the cloned cDNA (Fig. 6C, lane 2).

Sequence analysis of L. infantum LNP18 genomic clones and comparison with the L. major LNP18 cDNA sequence revealed a putative initiation codon 39 nucleotides immediately upstream of the codon proposed for L. major LNP18. Since the open reading frames of the two genes are identical, we cannot eliminate the possibility that this in-frame ATG could be the translation start codon for both genes. In such a case the molecular mass of the deduced amino acid sequence would be slightly larger (11 kDa; 111 amino acids with a pI of 11.74) than the predicted Mr, of L. major (9.9 kDa). This analysis further supports the hypothesis that the proteins detected are the products of the LNP18 gene.

LNP18 expression was also investigated during in vitro growth of promastigotes from the logarithmic phase to the stationary phase (that is, as promastigotes differentiate from a noninfective stage to an infective stage) (Fig. 5C). An α-tubulin antibody was used to examine whether LNP18 is differentially expressed during parasite differentiation. The housekeeping gene is down-regulated twofold in stationary-phase promastigotes compared to log-phase promastigotes (15). Densitometry analysis of both α-tubulin bands and LNP18 bands for the two developmental stages revealed ratios of 3.5 (lane 2 versus lane 1) and 1.7 (lane 1 versus lane 2), respectively. These data, combined with the known down-regulation of the α-tubulin protein, suggest that the LNP18 level increases threefold during promastigote maturation in culture.

The observed sequence similarity of LNP18 and H1 histones prompted us to investigate the possible nuclear localization of LNP18. Western blot analysis of Leishmania isolated nuclei (Fig. 5D) and staining of whole promastigotes by indirect immunofluorescence (Fig. 5E), when the anti-LNP18 antibodies were used as probes, showed that the protein is located exclusively in the nucleus (Fig. 5D, lane 1). To obtain a better idea of the LNP18 localization, we performed confocal microscopy (Fig. 5F). No protein was detected in the cytoplasmic extracts (Fig. 5D, lane 2) or the cytoplasm of whole parasites (Fig. 5E and F) with either approach. Ponceau S staining of membranes after protein transfer confirmed that the amounts of protein transferred to nitrocellulose were equivalent (data not shown).

In vitro UV-cross-linking hybridization: detection of an LNP18–DNA complex. In order to demonstrate that LNP18, which has characteristics of a DNA-binding protein, does interact with Leishmania DNA, we used the UV-cross-linking hybridization technique (41). Nuclear extracts were irradiated as described in Materials and Methods. UV irradiation induces cross-links between nucleic acids and proteins in close contact. This method has an additional advantage, its specificity. Thus, boiling extracts from irradiated cells in the presence of SDS causes the dissociation of any nonspecific nucleic acid–protein complexes. After UV treatment, samples were separated by SDS-PAGE and analyzed by Western blotting. Hybridization of the blot with the anti-LNP18 antibodies revealed an approximately 94-kDa band (Fig. 7, lane 4). In contrast, the 94-kDa band was not detected when samples were treated with DNase I prior to UV treatment and when nonirradiated nuclear control samples were used (Fig. 7, lanes 2 and 1, respectively). Treatment with DNase I after UV cross-linking slightly affected the migration of the 94-kDa complex. Most probably the resulting 90-kDa band (lane 3) corresponds to a DNA-protein complex. Hence, by using this simplified method which induces
FIG. 5. (A) Gel electrophoresis and immunoblotting of *L. major*, *L. infantum*, and *L. amazonensis* promastigotes (lanes 1 to 3, respectively) probed with antibodies raised against the recombinant protein (anti-rLNP18). The positions of molecular size standards are indicated on the left. (B) Western blot analysis of lysates from lesion-derived amastigotes (lane 1) and promastigotes (lane 2). Lysates were separated by SDS-15% PAGE, electroblotted, and probed with anti-rLNP18 and α-tubulin antibodies (NeoMarkers). The positions of molecular size standards are indicated on the left. (C) Western blot analysis of *L. major* promastigotes grown to the early logarithmic phase (day 3) (lane 2) and the stationary phase (day 5) (lane 1). The blot was probed with anti-rLNP18 and α-tubulin antibodies. (D) *L. major* isolated nuclei and cytoplasmic extracts from the same number of parasites (lanes 1 and 2, respectively). SDS-PAGE was performed under reducing conditions on 12.5% polyacrylamide gels. The positions of molecular size standards are indicated on the left. (E and F) Immunofluorescence labeling of promastigotes when the anti-rLNP18 antibodies were used as probes. *L. major* promastigotes (1.5 × 10^6 cells/ml of PBS) were fixed on glass microscope slides. Binding of anti-rLNP18 in the nucleus was visualized by using a secondary antibody conjugated with fluorescein isothiocyanate (FITC) and either phase-contrast microscopy performed with a photomicroscope (E) or confocal laser microscopy (F). For confocal microscopy an overlay image is also shown.
cross-links between nucleic acids and proteins in close contact, we showed that LNP18 is a potential DNA-binding protein.

Modulation of LNP18 level. Together, our data indicate that LNP18 has interesting features that make its function implicit. Therefore, we developed L. major transfectants in which the endogenous level of LNP18 was modulated. This was accomplished by transfecting L. major promastigotes with a plasmid construct developed by cloning the cDNA of LNP18 in the sense orientation into the SmaI restriction site of the pX63-HYG polylinker. Transfectants with the LNP18 cDNA and transfectants with the vector alone, developed in parallel, were selected in medium 199 with hygromycin B at a final concentration of up to 500 μg/ml. The integrity of the plasmids in these transfectants was confirmed by restriction mapping and sequencing, as well as by Southern blot analysis with specific probes (Fig. 6A). As expected, semiquantitative RT-PCR performed with RNA isolated from the transfectants showed significantly higher levels of LNP18 RNA in the pX63-HYG–LNP18 transfectants than in the transfectants with the vector alone (Fig. 6B, lanes 2 and 4, respectively). The level of LNP18 protein was significantly increased (approximately four- to fivefold) in the pX63-HYG–LNP18 transfectants compared to the control (Fig. 6C). This was estimated by densitometry analysis of the intensity of the LNP18 18-kDa–16-kDa doublet in controls and transfectants overexpressing LNP18 compared with the intensity of α-tubulin.

Infection of mouse macrophages. Macrophages (cell line J774) cultured in 96-well plates were infected at a ratio of 10 parasites per cell for 5 h with stationary-phase wild-type strain LV39 promastigotes and with LNP18-overexpressing and control plasmid-containing transfectants. The growth rate of amastigotes within macrophages was subsequently monitored. There was no difference in the ability to invade macrophages, the number of infected macrophages, or the number of intracellular parasites during the first 24 h of infection. This was evaluated by cytopsin and Giemsa staining of cells (data not shown). However, as shown in Fig. 8A, the number of amastigotes released from macrophages (transformed into promastigotes when they were incubated at 26°C for 48 h) infected with the LNP18 transfectants was significantly lower (sevenfold lower) than the number of parasites released from macrophages infected with promastigotes containing the control plasmid and with wild-type promastigotes. Similar results were obtained in three separate experiments performed with parasites generated in two transformation experiments.

It should be noted that amastigotes released from macro-
phages transformed into promastigotes at 26°C grew in the presence of hygromycin B, indicating that they retained the plasmid. These results suggest that LNP18 affects intramacrophage survival and/or replication of these parasites. The infection rates of the transfectants were also evaluated by flow cytometry. Amastigote-containing macrophages were detected with a polyclonal mouse antibody raised against amastigotes isolated from lesions of infected BALB/c mice and purified by low-pH elution of antibodies from strips blotted with amastigote lysates. By using this approach the infection rates in *L. major*-infected macrophages were measured with a high degree of specificity and reproducibility. The histogram in Fig. 8B clearly shows two well-defined populations corresponding to non-amastigote-containing cells and infected cells. The flow cytometric assay could not separate cells containing a single parasite from cells containing more than one amastigote. The accuracy and reproducibility of flow cytometry for detection of intracellular amastigotes, as well as tests for the efficacy of drugs against intracellular parasites, were recently examined in two independent studies (18, 27). The results of four independent experiments showed that the infectivity of the transfectants overexpressing LNP18 was significantly lower than that of the control plasmid-containing transfectants; the number of infected cells differed by threefold. The infectivity of the latter (approximately 35 to 40% of macrophages contained at least one amastigote) was comparable to that of wild-type parasites. The results obtained with both approaches strongly suggest that the level of expression of LNP18 modulates parasite infectivity.

**DISCUSSION**

In this paper we describe identification and characterization of a novel two-copy gene of *Leishmania* that encodes a developmentally regulated nuclear protein, designated LNP18. LNP18 is a DNA-binding protein that shows sequence similarity with H1 histones of trypanosomatids and, in particular, with histone H1 of *L. major*. It is recognized as a 18-kDa–16-kDa protein doublet which is highly conserved in the genus *Leishmania*. Both polypeptides appear to be related to LNP18 since...
analysis of transfectants overexpressing the gene showed that both polypeptides were up-regulated. Furthermore, the possibility that anti-LNP18 antibodies cross-react with the closely related L. major H1 protein must be excluded since the LNP18 antibodies do not seem to cross-react with the histone H1 protein in lesion-derived amastigotes (Fig. 5B, lane 1), in which the expression of H1, at both the mRNA and protein levels, is significantly higher than the expression of H1 in L. major promastigotes (21, 40). Like histone H1 polymorphism in L. major, LNP18 polymorphism may reflect the presence of variants possessing common epitopes, possibly as a result of post-transcriptional modification, a feature seen in H1 histones of higher eukaryotes (46).

Several lines of evidence suggest that functional significance underlies the heterogeneity of the H1 class of histones. In mice there are at least seven different H1 variants, which display different patterns of expression during development and differentiation (20). Thus, we investigated the expression profile of LNP18 during parasite growth and differentiation in vitro. The LNP18 level appeared to increase threefold as promastigotes differentiated from a non-infective stage to an infective stage and decreased several-fold in the amastigote stage, as assessed by Northern and Western blot analyses.

Infection of macrophages in vitro with transfectants overexpressing LNP18 showed that they are significantly less infective than the transfectants with the vector alone. These results suggest that the level of LNP18 expression modulates Leishmania infectivity. The mechanism by which the level of LNP18 expression affects Leishmania infectivity is not known yet. However, there is considerable evidence that histone H1 functions as a nonspecific repressor of transcription in higher eukaryotes and in Saccharomyces cerevisiae (36, 61). Similar observations have been made for the protozoan Tetrahymena, which is evolutionarily close to Leishmania. It has been shown that histone H1 is involved in the modulation of gene expression. Therefore, it is possible that LNP18 is involved in control of the expression of genes modulating Leishmania infectivity.

Recent findings also support the linkage of H1 expression with the cell cycle. In Trypanosoma brucei, H1 variants have been postulated to participate in the regulation of cell cycle progression and differentiation (3), as has been postulated for higher eukaryotes (9). Expression of LNP18, an H1-like protein, could be also linked to the cell cycle and gene expression and thus could control parasite differentiation and/or replication. Studies are in progress to define the functional role of this gene and protein in parasite growth and/or development. Moreover, the ability of transfectants to establish infections in vivo is currently being investigated.

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