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Expression and subcellular localization of ORC1 in *Leishmania major*

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ABSTRACT

The mechanism of DNA replication is highly conserved in eukaryotes, with the process being preceded by the ordered assembly of pre-replication complexes (pre-RCs). Pre-RC formation is triggered by the association of the origin replication complex (ORC) with chromatin. *Leishmania major* appears to have only one ORC ortholog, ORC1. ORC1 in other eukaryotes is the largest of the ORC subunits and is believed to play a significant role in modulating replication initiation. Here we report for the first time, the cloning of ORC1 from *L. major*, and the analysis of its expression in *L. major* promastigotes. In human cells ORC1 levels have been found to be upregulated in G1 and subsequently degraded, thus playing a role in controlling replication initiation. We examine the subcellular localization of *L. major* ORC1 in relation to the different stages of the cell cycle. Our results show that, unlike what is widely believed to be the case with ORC1 in human cells, ORC1 in *L. major* is nuclear at all stages of the cell cycle.

DNA replication in eukaryotes involves firing of multiple origins in S phase, preceded by origin selection in G1 phase. Origin selection is marked by the assembly of multi-protein pre-replication complexes (pre-RCs). Pre-RC formation is initiated by the binding of a six subunit complex ORC (origin recognition complex) to origin DNA, followed by the ordered recruitment of additional proteins Cdc6, Cdt1 and Mcms2-7 [1–3]. With the onset of S phase, cyclin-dependant kinases and Cdc7–Dbf4 kinase target the pre-RC proteins and essential replication factors, such as Cdc45, initiating a series of events culminating in DNA synthesis [1,4–7]. Association of Cdc45 marks origin activation as it is responsible for recruiting the DNA polymerase alpha-primase complex [5,8]. The involvement of ORC in replication initiation was first described in *Saccharomyces cerevisiae*. ScORC, containing six subunits Orc1p–Orc6p, interacts with replication origins in an ATP-dependent manner [9]. ORC-mediated ATP hydrolysis is essential for recruiting MCM proteins [10]. ORC orthologs have been identified in many eukaryotes like *Schizosaccharomyces pombe*, *Drosophila melanogaster*, *Xenopus laevis*, and *Homo sapiens*. Genetic and biochemical investigations demonstrate the ORCs of these organisms to be essential for DNA replication initiation (reviewed in [1]).

While extensive studies have analyzed pre-RCs in metazoans and fungi, less is reported about the pre-replication and replication apparatus in protozoans. In *Plasmodium falciparum* DNA pol α, DNA pol δ, PCNA, RPA1, ORC1, and MCM4 are expressed during those stages of the asexual phase where DNA replication occurs [11–15]. PfORC1 and PfMCM4 expression is also reported in the gametocyte stages [16,17]. PfORC1 and PfORC5 have been shown to colocalize with PfPCNA in replication foci [18]. In *Tetrahymena thermophila* an ORC-like protein complex containing an integral RNA subunit is largely responsible for rDNA origin recognition and interaction [19]. A novel protein, TIFI, is also implicated in modulating the replication of its DNA [20].

Though the replication machinery of *Leishmania* resembles that of higher eukaryotes, significant differences are implicated from the analysis of its genome [22] which reveals the presence of only one ORC ortholog—ORC1, orthologs of MCMs2-7 and Cdc45. No orthologs of Cdt1, MCM10, Dbf4, or Cdc7 are apparent. In *P. falciparum* too only orthologs of ORC1, ORC2, and ORC5, and MCMs2-7 orthologs appear to be present. Cdc6, Cdc45, and Cdt1 have not been found. The absence of several eukaryotic orthologs of pre-RC proteins in *Leishmania* suggests that in this organism unidentified proteins may be involved in replication initiation.

The ORC1p subunit in higher eukaryotes is the largest subunit. A role for human ORC1p in cell cycle control has been implicated [23–26]. The present study is the first report examining a pre-RC protein in any of the trypanosomatids. We report the cloning, overexpression and purification of ORC1 from *Leishmania major* (LmORC1) and demonstrate that LmORC1 is expressed in actively proliferating *L. major* promastigotes. We have examined the subcellular localization of LmORC1 in promastigotes at different stages of the cell cycle, and our results indicate that the protein remains nuclear throughout the cell cycle.

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Table 1
Sequences of oligonucleotides used in this study

Name	Sequence
ORC-F	5′-GAA T TC ATC ATA TGA AGC GGA GCC GGC GAG CG-3′
ORC-R	5′-GAA TTC GGA TCC TCA CAA GTG CAG CGC TGC C-3′
ORC-RR	5′-TCA CAA GTG CAG CGC TGC C-3′
ORC-RT-F	5′-TTA CTG TGG CTG TCC TCG G-3′
ORC-RT-R	5′-CCT CCT TGG CGT CAA GAA G-3′
ORC-GFP-F	5′-TCG GAT CCA CCA TGA AGC GGA GCC GGC GAG CG-3′
ORC-GFP-R	5′-TCG GCC GGC ACG GCC TCC CAA GTG CAG CGC TGC C-3′

Materials and methods

Leishmania culture. Leishmania major promastigotes were cultured in M199 medium supplemented with 10% FCS, 2 mM L-glutamine, 100 μM adenine, and 5 μg/ml hemin.

Genomic DNA isolation. Harvested cells (5×10^8) were lysed in 10 mM Tris–Cl (pH 8.0), 10 mM EDTA, 150 mM NaCl, and 1% SDS. Genomic DNA was precipitated with ethanol following proteinase K (Roche) treatment (100 μg/ml) and phenol:chloroform extraction.

RNA isolation. Total RNA was isolated from 5×10^7 promastigotes in the logarithmic phase of growth using the TRIzol Plus RNA Purification Kit (Invitrogen, USA).

RT-PCR analysis. cDNA was synthesized after treating 1–2 μg total RNA with 50 U DNase I (Fermentas), using Mu-MLV reverse transcriptase (Fermentas). One-tenth of the cDNA synthesis reaction was analyzed by PCR.

Cloning of ORC1. ORC1 gene was amplified with end-primers ORC-F and ORC-R (Table 1) using DNA polymerases Phu (Finnzymes) and Pfu (Stratagene), and cloned into the SmaI site of pUC18. For overexpression the gene was subcloned into expression vectors pASK-IBA43plus (IBA BioTAGnologies) (BamHI–PstI sites), pThioHisB (Invitrogen, USA) (NcoI–EcoRI sites) and pGEX-4T2 (GE, USA) (EcoRI–NotI sites).

Overexpression and purification of ORC1 protein. BL21 CodonPlus cells harboring plasmids pASK-ORC1, pThio-ORC1, and pGEX-ORC1 were grown to mid-log phase and ORC1 expression induced with 0.1 mM IPTG. Cells were further incubated at 16 °C for 16–18 h. To assess protein solubility, harvested cells were resuspended in PBS, lysed on ice by sonication, and the lysate clarified by centrifugation (10,000*g*). The pellet was solubilized in 3× SDS-sample loading buffer. Lysate and pellet were analyzed for ORC1. To obtain purified ORC1, ORC1 was solubilized from the pellets obtained after clarification of sonicated cell lysates, using 8 M urea in PBS containing 100 mM NaCl. The solubilized protein was dialyzed step-wise against decreasing concentrations of urea. The protein that precipitated out was collected by centrifugation (10,000*g*) and resolubilized in 3× SDS-sample loading buffer.

Raising antibodies to ORC1 and their characterization. Purified protein was resolved on SDS–PAGE, ORC1 band excised, crushed, and suspended in PBS for immunization of rabbits. Western blotting was done by standard methodology [27].

Preparation of Leishmania nuclear extracts. Leishmania nuclear extracts were prepared using the NE-PER kit (Pierce Biotechnologies).

Immunofluorescence analysis. LmORC1 was amplified using primers ORC-GFP-F and ORC-GFP-R (Table 1) and subcloned into BamHI–SfiI restriction sites of pXG−/GFP+ [28]. pXG−/GFP+/ORC was transfected into *Leishmania* promastigotes by electroporation as described [29,30]. Promastigotes harvested 24–40 h post-transfection were washed with PBS, fixed with 2% paraformaldehyde, and cell spreads made on polylysine coated cover-slips. Mounting was in anti-fade solution containing DAPI (Vectashield, Vecta Laboratories). Cells were viewed and images acquired using a

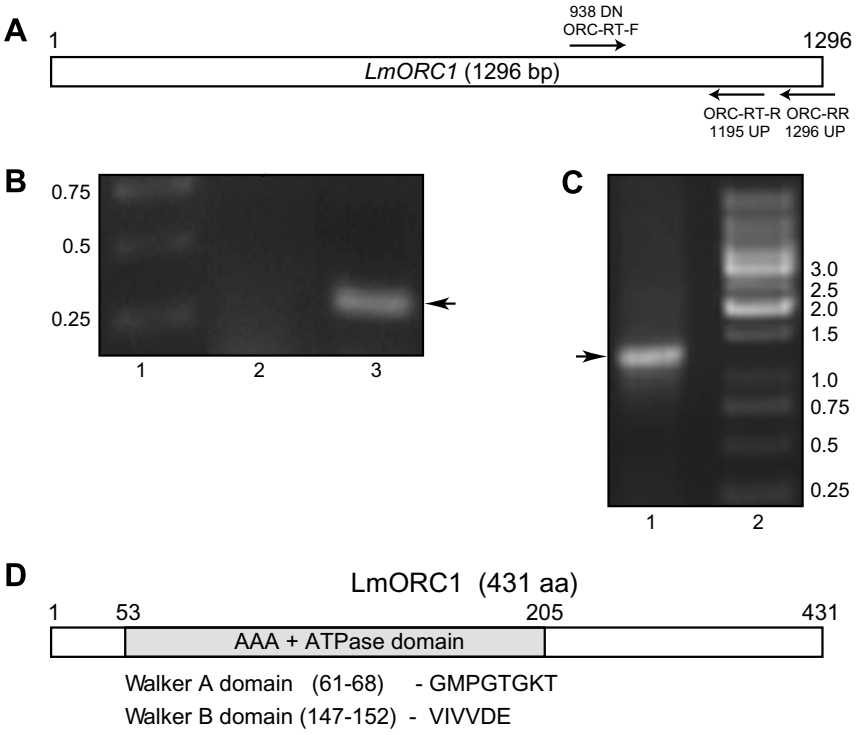

Fig. 1. Cloning of *Leishmania major* ORC1. (A) RT-PCR was performed using primer ORC-RR (Table 1), an ORC1 end-primer, to make cDNA. A section of the cDNA was then amplified using primers ORC-RT-F and ORC-RT-R (Table 1). (B) RT-PCR yielded the expected ~ 250 bp product. Lane 1—DNA marker in kb; lane 2—control PCR reaction performed on RNA that had not been reverse transcribed, to rule out amplification due to genomic DNA contamination; lane 3—RT-PCR shows specific ORC1 product. (C) *ORC1* was cloned by amplification from *Leishmania major* genomic DNA. Lane 1—~1.3 kb *ORC1* gene product. Lane 2—DNA marker in kb. (D) The ORC1 protein is 431 amino acids in length. It has a putative AAA+ ATPase domain between residues 53–205. Positions of Walker A and Walker B domains present in the protein are indicated.

100× objective, utilizing a motorized epifluorescence microscope (Upright Axioimager M1; Carl Zeiss MicroImaging, Inc.) equipped with a high-resolution camera (AxioCam MRm Rev. 2; Carl Zeiss MicroImaging, Inc.). Images were analyzed by AxioVision Software Rel. 4.4 (Carl Zeiss MicroImaging, Inc.).

Results and discussion

Cloning and purification of ORC1

Leishmania major whole genome sequence revealed the presence of a single ORC ortholog—ORC1 [22]. The present study was initiated with the analysis of *Leishmania* RNA for *ORC1* transcripts as the first step towards determining if the gene was being expressed in the organism. cDNA was made using an ORC specific primer ORC-RR (Table 1 and Fig. 1A) designed based on the published genome sequence. A section of the cDNA so synthesized was amplified using primers ORC-RT-F and ORC-RT-R (Table 1 and Fig. 1A), also designed based on the published genome sequence. The RT-PCR product was the expected ~250 bp in size (Fig. 1B). The PCR product was sequenced to confirm its authenticity. Having ascertained that the gene was being transcribed, the full length *ORC1* gene was cloned from *L. major* genomic DNA, as, like most *Leishmania* genes, it has no intron. The ~1.3 kb amplicon so obtained (Fig. 1C) was cloned into pUC18 and completely sequenced using overlapping primers. Clones of amplicons obtained using each of the two proof-reading enzymes were sequenced. The sequence matched with the published genome sequence.

Amino acid sequence analysis of LmORC1 reveals that the protein has a AAA+ ATPase motif (residues 53–205) (Fig. 1D). The presence of Walker A and Walker B domains indicates that the protein most likely binds ATP, and suggests that ATP hydrolysis by the protein may play a functional role in the assembly of pre-RCs in this organism. *S. cerevisiae* ORC binds origins in an ATP-dependent manner [9]. Though ORC mediates ATP hydrolysis in *S. cerevisiae* and *D. melanogaster* it is not essential to DNA binding [31,32]. Human ORC1 and PfORC1 also demonstrate ATPase activity [15,33].

To assess ORC1 expression in *Leishmania* at the protein level we raised antibodies to the recombinant protein. The gene was subcloned into three *Escherichia coli* expression vectors—pASK-IBA43-plus, pThioHisB, and pGEX-4T2. In all cases the protein was overexpressed upon induction (Fig. 2A), but was largely insoluble

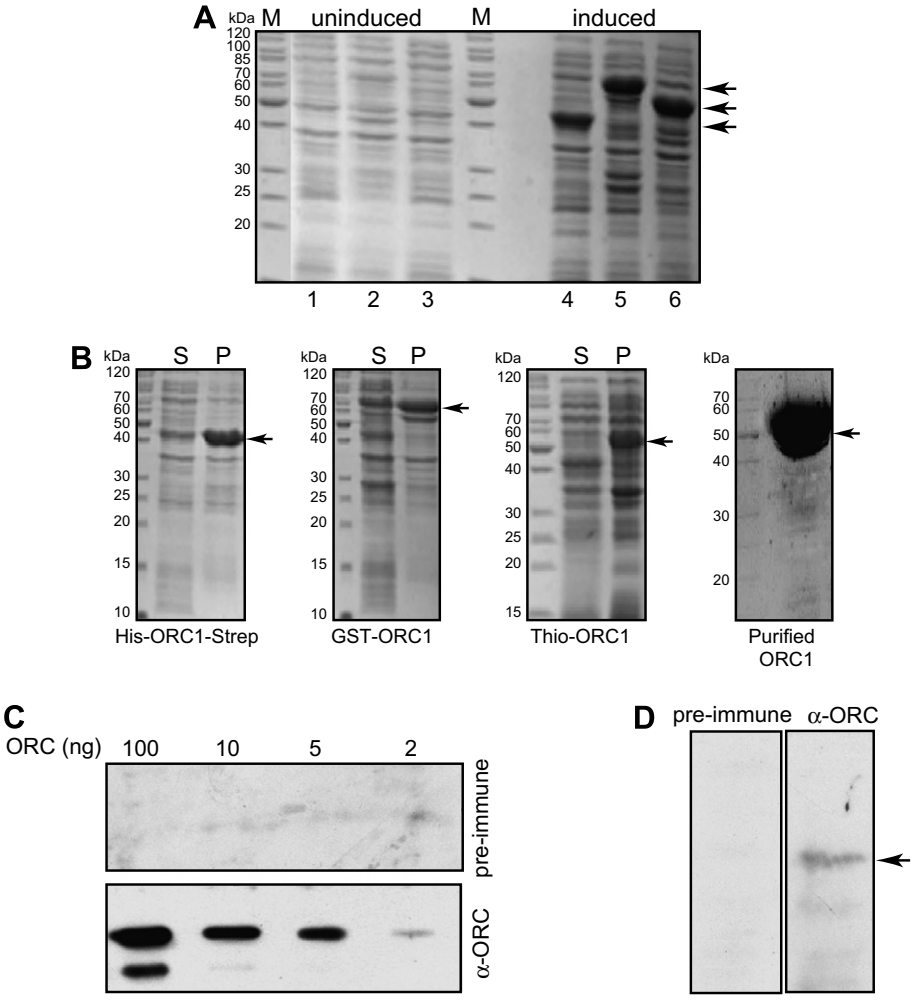

Fig. 2. Expression of LmORC1 in *Leishmania major* promastigotes. (A) Overexpression of recombinant LmORC1 in *E. coli.* Lanes marked 1 and 4—His-ORC1-Strep (overexpression from plasmid pASK-ORC1); lanes marked as 2 and 5—GST-ORC1 (overexpression from plasmid pGEX-ORC1); lanes marked as 3 and 6-Thio-ORC1 (overexpression from plasmid pThio-ORC1). Overexpressed protein bands are indicated with arrows. (B) Overexpressed His-ORC1-Strep, GST-ORC1 and Thio-ORC1 (indicated by arrows in the first three panels) were all largely insoluble. The fourth panel shows purified ORC1 protein that was used to raise antibodies. S-soluble fraction, P—pellet fraction. (C) Anti-ORC1 antibodies used in standard Western blot analysis of recombinant His-ORC1-Strep protein detected as low as 2 ng protein when used at a dilution of 1:500. Pre-immune serum used in identical control Western blots did not detect anything. (D) Analysis of *Leishmania major* nuclear extracts by standard Western blotting with the anti-ORC1 antibodies used at dilution of 1:500 revealed that ORC1 is expressed in *Leishmania*. Approximately 80 μg extract was analyzed by Western blotting. Pre-immune serum used as a control in an identical Western blot analysis did not detect anything.

even when overexpressed at low temperatures (Fig. 2B). To raise polyclonal antibodies we utilized recombinant ORC1 expressed from pASK-ORC1 as it had the shortest tags. Recombinant ORC1 was solubilized using 8 M urea. In attempting to refold the protein by step-wise dialysis against decreasing concentrations of urea, LmORC1 precipitated out of solution. The precipitated protein was resolubilized in 3× SDS-sample loading buffer and analysis of this resolubilized protein revealed it to be more than 99% pure (Fig. 2B). The protein was confirmed to be LmORC1 by LC–MS analysis (data not shown). This protein was used to raise antibodies in rabbits.

ORC1 is expressed in actively dividing Leishmania promastigotes

The specificity and efficacy of the antibodies obtained were tested against recombinant LmORC1. The antibodies detected as less as 2 ng recombinant LmORC1 in Western blot analyses. Pre-immune serum did not react with equivalent amounts of the protein (Fig. 2C). ORC1 expression in *Leishmania* was analyzed in nuclear extracts made from exponentially growing promastigotes. Results from Western blot analysis (Fig. 2D) indicate that LmORC1 is expressed in actively dividing promastigotes.

Subcellular localization of ORC1

ORC1 is believed to be the only ORC whose expression is regulated in a cell cycle specific manner in higher eukaryotes. Though some studies indicate that ORC1 is expressed throughout the cell cycle [34–36], others have demonstrated that it is the only ORC subunit whose expression levels are regulated with the progress of the cell cycle [23–26]. The protein has been shown to be upregulated in G1 phase, but gets degraded in S phase. The expression levels of the other ORC subunits are not modulated by cell cycle progression [25]. The regulation of ORC1 in a cell cycle-dependent manner is believed to be a significant feature in the regulation of cell cycle progression. As *Leishmania* has only one ORC subunit, it was of particular interest to us to see how this protein is regulated. *Leishmania* cells can be synchronized using hydroxyurea [37] but this blocks cell cycle progression in early S phase. To be able to analyze G1 cells as well as S phase and G2/M phase cells for LmORC1 expression we adopted the approach of immunofluorescence analysis of asynchronous cultures. The kinetoplast in trypanosomatids as seen by immunofluorescence upon DAPI staining can be used as a marker for cell cycle stage of an individual cell [38]. This is due to the staggered timing of DNA replication of kinetoplast versus genome. Thus, cells in G1 phase contain one nucleus and one short roundish kinetoplast. Cells in S phase contain one nucleus and one elongated kinetoplast. Cells in G2/M contain one nucleus and two kinetoplasts, as by this phase of the cell cycle not only is kinetoplast replication complete, but also the newly synthesized kinetoplasts are already separate. Post-mitotic cells have two completely separated nuclei and two kinetoplasts. Our attempts to examine LmORC1 in individual cells at different stages of the cell cycle by this methodology, using the anti-ORC antibodies we raised, in indirect immunofluorescence studies,

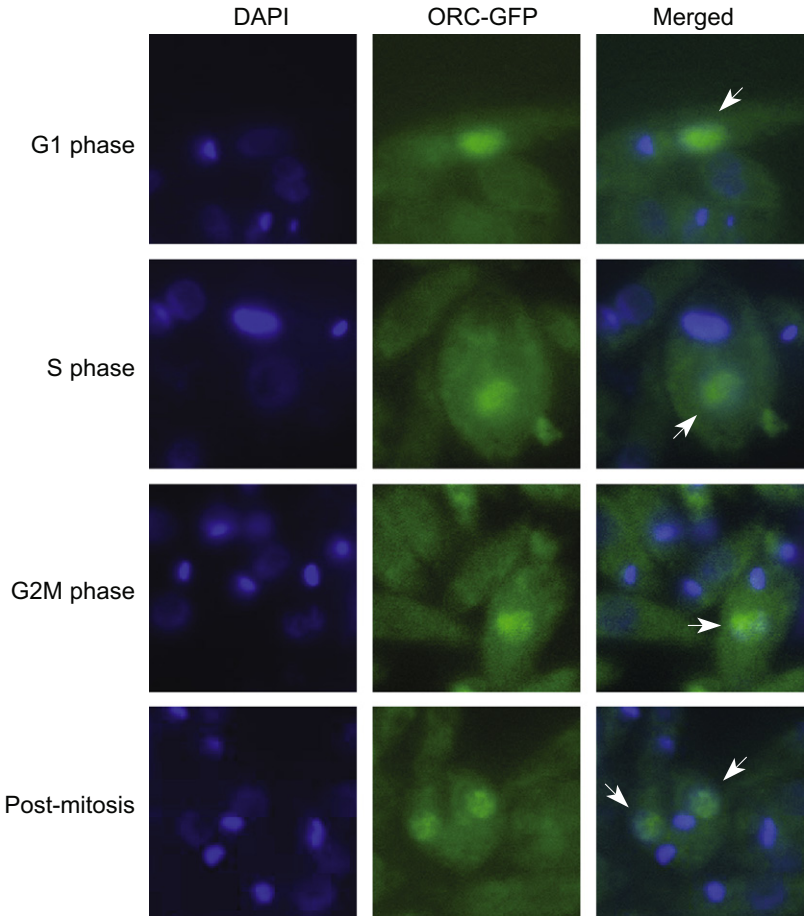

Fig. 3. ORC1 remains nuclear throughout the cell cycle. G1 phase cells—one nucleus, one short kinetoplast. S phase cells—one nucleus, one elongated kinetoplast. G2/M phase cells—one nucleus, two kinetoplasts. Post-mitosis—two nuclei, two kinetoplasts.

met with failure. The antibodies did not work in either immunoprecipitation or immunofluorescence experiments. We therefore attempted to look at ORC1 expression and subcellular localization in individual cells by fusing ORC1 to GFP and examining direct fluorescence of ORC1-GFP. Accordingly, ORC1 was expressed in fusion with GFP at its C-terminal end using *Leishmania* expression vector pXG−/GFP+ [28], a gift from Prof. S.M. Beverley. *Leishmania* promastigotes were transfected with plasmid pXG−/GFP+/ORC. Our attempts to make a stable cell-line harboring this plasmid using G418 selection failed, suggesting that high levels of expression of this protein are detrimental to survival. For immunofluorescence analysis, therefore, transiently transfected cells were examined. We analyzed transfected cells that were at different stages of the cell cycle. Contrary to our expectations, we found that ORC1-GFP was detected in the nucleus at all stages of the cell cycle (Fig. 3). While the levels of expression varied, from some cells with very high expression levels to cells with low levels of expression, the variable levels of expression did not correlate with specific stages of the cell cycle and seem to be more the effect of variable copies of plasmid getting transfected into the different cells. Fig. 3 shows cells with typical levels of expression in most of the cells. The continued detection of ORC1 at all stages of the cell cycle is unlikely to be the consequence of overexpression, as, in transiently transfected human cells, ORC1-GFP has been demonstrated to localize to the nucleus only in cells that are in G1 phase [26].

While cell cycle-dependent alterations in expression and subcellular localization of ORC1 as a mode of regulation of replication initiation is an attractive hypothesis, it appears not to be the case here. ORC1, though nuclear throughout the cell cycle, may be chromatin-bound only at certain stages. Studies in mammalian cells have yielded conflicting results. While some investigators report ORC1 to be bound to chromatin only in G1 phase [23,36], others demonstrate it to remain chromatin-bound throughout the cell cycle [35,39]. In *S. cerevisiae* and *S. pombe* the ORCs remain bound to origins throughout the cell cycle [40,41]. LmORC1 may be also be regulated by differential phosphorylation status of the protein at different stages of the cell cycle, which may impact the recruitment of the other proteins that are part of pre-RCs. Pre-RC proteins other than ORC1 are also likely to be responsible for controlling replication timing. Furthermore, considering the fact that several pre-RC proteins found in higher eukaryotes are missing here, it is entirely possible that novel Leishmania proteins may be involved in this process.

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