DNA replication is a complex multistep process that is comprised of initiation, elongation, and DNA damage repair. Chromosomal replication initiates with the assembly of the prereplication complex (pre-RC) at DNA sites along the chromosomes that are called origins of replication (27). In eukaryotes, the pre-RC is composed of an origin recognition complex (ORC) containing six proteins, Orc1 to Orc6, two proteins named Cdc6 and Cdt1, and the minichromosome maintenance (MCM) complex, which is composed of Mcm2 to Mcm7 proteins. The assembly of the pre-RC on chromatin occurs through the ATP-dependent binding of the heterohexamer ORC to chromatin (reviewed in references 4 and 36). Chromatin-bound ORC recruits Cdc6, an AAA+ ATPase protein with significant sequence similarity to Orc1 (5, 34). The ATP binding domain of Cdc6 is essential for pre-RC assembly (42, 53). The ATPase activity regulates the selection of specific DNA sequences known as origins of replication (47). Cdt1 physically associates with Cdc6 (29) and is also required for DNA replication in a wide range of organisms. Since Cdt1 also binds directly to the MCM complex, it may act as a chaperone for bringing the MCM proteins to the origin (reviewed in references 36 and 48). Therefore, available evidence indicates that ORC, Cdc6, and Cdt1 act together to allow the assembly of the heterohexamer MCM complex, whose helicase activity is essential for replication (23). As long as the pre-RC composed of ORC, Cdc6, Cdt1, and MCM is organized on the chromatin, origins become licensed to replicate. In addition, other proteins must associate with the origin prior to the successful initiation of DNA synthesis. The binding of regulatory factors and components of the replication fork to DNA allows origin unwinding, the recruitment of replicative DNA polymerases, and finally the establishment of replication forks (4, 48).

DNA replication must be carefully coordinated with the events of the cell cycle to ensure the stable maintenance of the genome. In eukaryotes, this is achieved by the assembly of the prereplication machinery at the G1 phase of the cell cycle, while the ability to license new replication origins is downregulated before entry into S phase. Since ORC, Cdc6, and Cdt1 are required for loading MCM onto the DNA but are not required for the continued MCM-DNA interaction (8, 15, 17, 44), the downregulation of their expression and/or activity at the end of G1 represents an effective way to block DNA replication (7). In Saccharomyces cerevisiae, ORC subunits are bound to the chromatin throughout the cell division cycle (48), but Cdc6 is phosphorylated by cyclin-dependent kinases (CDK) and degraded by proteolysis at the onset of the G1-S transition (16, 20). Cdc6 transcription is also regulated during the cell cycle, reaching its maximum expression in late mitosis and early G1 (35, 56). Similar to Cdc6, Cdt1 levels might be controlled by CDK-dependent transcription and proteolysis (40). In addition, MCM are exported from the nucleus during S phase, G2, and early mitosis, preventing the license of new origins in non-S-phase stages (39). In mammalian cells, the
Orc1 subunit is degraded in S phase by a polyubiquitinylation reaction that is CDK dependent (37). Unlike the process found in yeast, the mammalian Cdc6 remains bound to chromatin during the cell cycle. Therefore, the main way by which mammalian cells restrict replication to S-phase cells is by Cdt1 activity, which is regulated by geminin, a Cdt1 inhibitory protein (7).

Unlike other eukaryotes, little is known about the DNA replication process in trypanosomes, protozoan parasites that appear early in the evolution of eukaryotes. Genomic databases of trypanosomatids show that these organisms contain all McM proteins, but they do not contain sequences in their genome that could code for the ORC subunits, Cdc6, or Cdt1. Instead, they appear similar to archaean species that have one or more copies of proteins whose sequences are highly related to those of Orc1 and Cdc6 (often called the Orc1/Cdc6 protein) (12–14). Most of these organisms present a homohexameric McM, composed by just one subunit, and there is no sequence codifying to Cdt1 in their genome. However, recently, a candidate for the archaean initiator protein that is distantly related to eukaryotic Cdt1 was described (43). Trypanosomatids have a candidate gene for only one of the six subunits of the ORC, Orc1, which is also homologous to Cdc6. It is annotated as Orc1 (21), and we named it Orc1/Cdc6 here. As trypanosomatids have peculiar characteristics, with their genes transcribed in polycistronic units and processed by a trans-splicing reaction and with gene expression controlled mainly at the posttranscriptional level (11, 41, 51), we hypothesize that new strategies to deal with the regulation of replication and transcription processes can be found in these organisms. In the present work, we ask whether the Orc1/Cdc6 from Trypanosoma cruzi (TcOrc1/Cdc6), the agent of Chagas disease, and from Trypanosoma brucei (TbOrc1/Cdc6), the agent of sleeping sickness, are indeed involved in replication. We found that the genes encoding these proteins are expressed in both T. cruzi and T. brucei and that both recombinant TcOrc1/Cdc6 (rTcOrc1/Cdc6) and TbOrc1/Cdc6 present ATPase activity that increases in the presence of unspecific DNA. TcOrc1/Cdc6 and TbOrc1/Cdc6 proteins replace yeast Cdc6 in thermosensitive yeast mutants. Also, induction of Orc1/Cdc6 silencing by RNA interference (RNAi) in T. brucei results in enucleated cells. TcOrc1/Cdc6 and TbOrc1/Cdc6 are restrained to the nuclear space during the entire cell cycle and remain bound to DNA throughout the cell division cycle. These data show that Orc1/Cdc6 is a component of the prereplicative machinery in trypanosomes and that, in these organisms, the restriction of replication to one round during the cell cycle is not related to Orc1/Cdc6 expression, localization, or ability to bind to chromatin.

**MATERIALS AND METHODS**

**Parasites and growth conditions.** T. cruzi epimastigotes (Y strain) were cultured in liver infusion tryptose medium supplemented with 10% fetal bovine serum at 28°C (9). Procyclic forms of T. cruzi 427 (MITat 1.2) were grown in SDM-79 medium supplemented with 10% fetal bovine serum at 28°C (9). Procyclic forms of Trypanosoma brucei (TbOrc1/Cdc6), the recombinant protein was obtained by the induction of expression with 1.0 mM IPTG for 5 h at 37°C. Cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl (pH 8.0) and 300 mM NaCl supplemented with an EDTA-free complete protease inhibitor cocktail (Roche). The cell samples were passed through a French pressure cell (10,000 lb/in^2) in an ice bath. The insoluble rTcOrc1/Cdc6 was washed and solubilized in 8 M urea containing 500 mM NaCl and 20 mM sodium phosphate (pH 8.0). TcOrc1/Cdc6 was then purified in a nickel column (Novagen). Washes were done in the same buffer at pH 6.0, and the elution was done at pH 4.0. Eluted rTcOrc1/Cdc6 was dialyzed against phosphate-buffered saline (PBS) and used to immunize rabbits. Immunoblots were performed using sodium dodecyl sulfate (SDS) extracts of 10^7 cells per lane, with serum from rabbit diluted 1:2,000, and detection of secondary antibodies was done by using an ECL kit (Amersham Biosciences) using standard protocols. Separated membranes were stained with Ponceau, and incubated with 5-35S- or 32P- immune or immunoglobulin G (IgG). Alternatively, the membranes were incubated with antibody against glyceraldehyde-3-phosphate dehydrogenase diluted 1:1,000 (46) as a control for the amount of protein. After incubation, the membranes were exposed together in the same X-ray film. The image is the scanned X-ray film.

To insert the TcOrc1/Cdc6 and TbOrc1/Cdc6 genes into pMALc2x (New England Biolabs), the genes were amplified from the T. cruzi CL-Brener strain and from T. brucei 427 with specific primers (primers 5'-AAAGGATCCCTTGAGTTTTGTTGAAG and 5'-CGGTCATTTG and 5'-AACGGATCTCAAACTTGAAGG and 5'-AAAACTTCTTCTAGAACAG for T. cruzi) and primers 5'-AAAGGATCTCAAACTTGAAGG and 5'-AAAACTTCTTCTTCTAGAACAG for T. brucei). The resulting plasmid was transferred into Escherichia coli Rosetta(D3), and the recombinant protein was obtained by the induction of expression with 1.0 mM IPTG for 5 h at 37°C. Cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl (pH 8.0) and 300 mM NaCl supplemented with an EDTA-free complete protease inhibitor cocktail (Roche). The cell samples were passed through a French pressure cell (10,000 lb/in^2) in an ice bath. The soluble rTcOrc1/Cdc6 and TbOrc1/Cdc6 were washed and solubilized in 50 mM Tris-HCl (pH 8.0) and 300 mM NaCl. TcOrc1/Cdc6 and TbOrc1/Cdc6 were then purified in an amylose resin column (New England Biolabs). Washes were done with the same buffer, and elution was done with the same buffer supplemented with 10 mM maltose.

**ATPase activity.** One microgram of rTcOrc1/Cdc6 or rTbOrc1/Cdc6 fused to maltose-binding protein or the same amount of maltose-binding protein alone (as control) were incubated with increasing concentrations of ATP in 50 mM Tris-HCl (pH 8.0) and 1 mM MgCl₂. After 1 h at 37°C, the reactions were stopped by the addition of 15% trichloroacetic acid for 10 min on ice. The released inorganic phosphate (P) was detected using 1% ammonium molybdate and 180 mM ferrous sulfate and measured by the method of Fiske and Subbarow (22). Samples were read at 660 nm, and the spontaneous hydrolysis of ATP was subtracted. In some reaction mixtures, 1 μg of rTcOrc1/Cdc6 or rTbOrc1/Cdc6 fused to maltose-binding protein or maltose-binding protein alone was incubated in the presence of 100 pmol of salmon sperm DNA and 10 mM ATP. The obtained experimental data were adjusted to a classical Michaelis-Menten hyperbolic equation by using software program OriginLab v.7.0.

**Yeast complementation assay.** Trypanosome genes were inserted into the pYES vector (Invitrogen) for complementation of yeast strains. Primers 5'-AAAGGATCCCTTGAGTTTTGTTGAAG and 5'-AAAGGATCCCTTGAGTTTTGTTGAAG were used to clone TcOrc1/Cdc6, and primers 5'-AAAACTTCTTCTTCTAGAACAG AAGG and 5'-AAAGGATCCCTTGAGTTTTGTTGAAG were used to clone TbOrc1/Cdc6. The resulting plasmids (yESTcOrc1/Cdc6 and yESTbOrc1/ Cdc6) were transformed into thermosensitive yeast mutants that were kindly provided by Oscar M. Aparicio (University of Southern California). Thermosensitive yeast mutants are able to grow at 32°C but are not able to grow at 37°C due to mutations in the cdc6 gene (OAy741 strain) (1) or the orc1 gene (OAy661 strain) (25). These strains were transformed with either the empty pYES vector (Invitrogen) or pYES containing TcOrc1/Cdc6 or TbOrc1/Cdc6 in the presence of.
of lithium acetate as described previously (26). After transfection, the strains were grown in selective medium plates (His, Trp, and adenine for strain OAy741 and His, Trp, Lys, and adenine for strain OAy661) at 23°C. The clones were then transferred to a selective medium at 37°C. Cell extracts were prepared as described previously (32) using cells growing at 23°C. One-milliliter samples of cells grown overnight were collected by centrifugation at 3,000 × g for 1 min. The pellets were resuspended in 100 μl of water and 100 μl of 0.2 M NaOH, and were then incubated for 5 min at room temperature. The cell extracts were pelleted again, resuspended in 50 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, incubated at 95°C for 5 min, and resolved by SDS-PAGE.

RNAi and FACS analyses. A fragment of the TbOrc1/Cdc6 gene was PCR amplified (with primers 5’-AAAAGGTACATGAGTTGTAAAG and 5’- AA AAAGCTTCATATCCTCGATG -3′) and inserted into the BamHI/HindIII sites of the pGEM-T vector (54). Procylic forms of T. brucei 29-13 cell line were then transfected by electroporation in 4-mm cuvettes, using a Bio-Rad Gene Pulser II electroporator with two pulses of 1,600 V and 25 μF. After 24 h, 5 μg/ml of phleomycin was added for the selection of transfected cells. The synthesis of double-stranded RNA was induced by adding 1 μg/ml of tetracycline to exponentially growing cultures at time zero and adding an additional 0.5 μg/ml of tetracycline on each subsequent day. Growth curves were obtained over a period of 8 days after the induction of RNAi. For fluorescence-activated cell sorting (FACS) analysis, the cells were fixed in 70% methanol, stained with propidium iodide, and analyzed by flow cytometry as described previously (19).

Immunofluorescence. The trypomastigones were washed with PBS, allowed to attach to glass slides, fixed with 2% formaldehyde in PBS for 20 min, and then permeabilized with 0.1% Triton X-100 in PBS containing 0.5% bovine serum albumin (PBS-0.5% BSA) and with the monoclonal antibody MAbAC (culture supernatant, which recognizes an unknown flagellar structure, diluted 1:2 in PBS-0.5% BSA). This antibody was raised by immunizing BALB/c mice with insoluble detergent extracts enriched in a cytoskeletal fraction of T. cruzi epimastigotes as described previously (45). The positive hybridomas were selected by immunofluorescence assays and cloned by limiting dilutions. For the competition assay, the anti-TcOrc1/Cdc6 diluted 1:200 in PBS-0.5% BSA was maintained in the presence of 10 μg of the recombinant protein rTcOrc1/Cdc6 for 30 min at room temperature. After that, the solution containing anti-TcOrc1/Cdc6 and rTcOrc1/Cdc6 was added to the slides. The cells were washed three times with PBS and incubated with Alexa Fluor 488-conjugated anti-rabbit immunoglobulin G and with Alexa Fluor 555-conjugated anti-mouse immunoglobulin G (Invitrogen) in PBS-0.5% BSA. The slides were washed again and mounted with Vectashield (Vector) in the presence of 10 μg/ml of 4,6-diamidino-2-phenylindole (DAPI).

RESULTS

Amino acid sequence and structural analyses. The yeast and metazoan pre-RC is composed of an ORC containing the six Orc proteins, Orc1 to Orc6, two proteins named Cdc6 and Cdt1, and the MCM complex, which is composed of Mcm2-Mcm7 proteins. On the other hand, the pre-RC from Archaea contains a single protein, Orc1/Cdc6, that recognizes the replication origin recruiting the helicase component, represented by a homohexamer MCM, depending on the archaeon species. Genomic databases of trypanosomatids show that these organisms contain all MCM proteins and in sufficient quantities to form the MCM heterohexamer, but they do not contain sequences in their genome that could code for the ORC subunits, Cdc6, or Cdt1. Instead, two sequences were found annotated in the DBGET database (www.genome.ad.jp/dbget/ -as Orc1 in the genome of T. cruzi. The TIGR Parasite Database (www.tigr.org) showed two sequences annotated as Orc1 (Tc00104705351159.20 and Tc0107053508239.10) in the genome of T. cruzi and one sequence annotated as Orc1 (Tb11.02.5110) in the genome of T. brucei. The two Orc1 protein sequences from T. cruzi are 98.2% identical, and inspection of the genomic environment of the two T. cruzi genes indicates that they are syntenic and likely represent alleles rather than distinct genes. The Orc1 proteins from T. cruzi and T. brucei are 77.1% and 77.8% identical. Since trypanosome Orc1 is also homologous to Cdc6 (21), Orc1 from trypansom was named Orc1/Cdc6 here. The primary sequences of both proteins contain the classical signal for the nuclear signal import in the N-terminal region (Fig. 1A). The primary sequences also contain Walker A and B motifs (related to the binding of ATP/GTP) and sensor I and II regions (involved in ATP hydrolysis), typical features of members of the prereplication machinery presenting the AAA + ATPase fold (28, 52). In order to gain further insight into the structure of trypansom Orc1/Cdc6, we submitted the primary sequences of TcOrc1/Cdc6 and TbOrc1/Cdc6 to the Phyre server for structural alignment, a web-based method for protein fold recognition using one- and three-dimensional sequence profiles coupled with secondary structure (30). Top significant hits indicated a higher structural similarity of trypanosome Orc1/Cdc6 to archael Orc1/Cdc6 structures. We compared the predicted secondary structures of trypanosome Orc1/Cdc6 with the secondary structure observed in the crystal structure of Aeropyrum pernix Orc1 (Fig. 1A). Besides confirming the presence of Walker motifs, we also found winged helix domains at the C terminus, which are structural motifs commonly used to bind nucleic acid sequences (24). However, it is important to note that the predicted structures in the trypansom winged helix domains are much shorter than those in the A. pernix Orc1. Moreover, there are more positive amino acids (that contact phosphate groups in DNA) in the archael winged helix than in the trypanosome domains.

Orc1/Cdc6 expression and ATPase activity. Since the results of in silico analysis of trypansom Orc1/Cdc6 suggest that it is involved in replication, we examined the presence of this protein in whole-cell extracts from epimastigotes, the replicative form of T. cruzi, and from the procyclic form of T. brucei. To raise specific antibodies against Orc1/Cdc6, the corresponding T. cruzi gene was amplified by PCR, cloned into the expression vector of lithium acetate as described previously (26). After transfection, the strains were grown in selective medium plates (His, Trp, and adenine for strain OAy741 and His, Trp, Lys, and adenine for strain OAy661) at 23°C. The clones were then transferred to a selective medium at 37°C. Cell extracts were prepared as described previously (32) using cells growing at 23°C. One-milliliter samples of cells grown overnight were collected by centrifugation at 3,000 × g for 1 min. The pellets were resuspended in 100 μl of water and 100 μl of 0.2 M NaOH, and were then incubated for 5 min at room temperature. The cell extracts were pelleted again, resuspended in 50 μl of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, incubated at 95°C for 5 min, and resolved by SDS-PAGE.
Cdc6 is expressed in both T. cruzi and T. brucei/Cdc6 were 48 kDa, we conclude that Orc1/Cdc6 from trypanosomes is homologous to Orc1 (ApeOrc1). The trypanosomatid sequences were then aligned by ClustalW and compared to the pdb structure of Orc1 using the Expresso tool from T-Coffee. The secondary structures of the trypanosomatid sequences were obtained from PSI-Pred (Fig. 1A). The color coding represents the agreement between the pairwise-aligned sequence as follows: blue tubes, α helices; arrows, β sheets. The numbers at the bottom of the panel provide the average consistency (cons) (0 to 100) for each sequence, thus indicating whether some sequences may have a less reliable alignment. (B) Epimastigote cells from T. cruzi or procyclic forms from T. brucei were boiled in the SDS-PAGE loading buffer, and the supernatants were analyzed by SDS-PAGE. Membranes were stained with Ponceau and then subjected to Western blotting using either anti-rTcOrc1/Cdc6 immune or preimmune serum.

Vector pET28a, and transformed into the Rosetta(DE3) strain of E. coli. After induction, the protein was expressed (see Fig. S1A in the supplemental material) and was found to be insoluble after bacterial lysis. His$_6$-rTcOrc1/Cdc6 was purified from inclusion bodies by solubilization in urea and chromatography on a nickel column (see Fig. S1B in the supplemental material), and the purified protein was used to immunize rabbits. The bottom bands present in the elution fraction are rTcOrc1/Cdc6 breakdown products, since the anti-TcOrc1/Cdc6 cannot recognize any band in the bacterial extract. The specificity of the generated antibodies was further checked by Western blot analysis using preimmune and immune sera against the purified proteins. We cloned both TcOrc1/Cdc6 and TbOrc1/Cdc6 in the pMAL vector, which favors recombinant protein solubilization. His$_6$-rTcOrc1/Cdc6 was purified from the Rosetta(DE3) strain containing rTcOrc1/Cdc6 and rTbOrc1/Cdc6, or the maltose-binding protein alone as control, were incubated with increasing concentrations of ATP. The released Pi was quantified (see Materials and Methods), and as shown in Fig. 2D, both rTcOrc1/Cdc6 and rTbOrc1/Cdc6 presented ATPase activities that follow a Michaelis-Menten kinetic model ($R^2 = 0.97$ for rTcOrc1/Cdc6 and $R^2 = 0.96$ for rTbOrc1/Cdc6) for the adjustment of both curves to the theoretical Michaelis-Menten hyperbolic function. As expected from the sequence similarities of the Walker motifs, the $K_{m}$ of both proteins for ATP were quite similar (the $K_{m}$ of rTcOrc1/Cdc6 is 438.60 μM ± 105.71 μM, and the $K_{m}$ of rTbOrc1/Cdc6 is 462.41 μM ± 309.73 μM). An assay using the maltose-binding protein alone presented no ATPase activity (Fig. 2E).

It has been proposed that in yeast the Cdc6 ATPase activity promotes origin DNA sequence specificity, since the Cdc6 ATPase promotes the dissociation of Cdc6 from the complex on DNA lacking origin activity. Therefore, we tested trypanosome Orc1/Cdc6 ATPase activities in the presence of DNA. Interestingly, when a nonspecific DNA (salmon sperm DNA) was added to the rTcOrc1/Cdc6 or rTbOrc1/Cdc6, ATPase activity increased (Fig. 2D), suggesting that, as found in yeast, trypanosome Orc1/Cdc6 ATPase activity might be involved in the selection of specific sequences.

**Trypanosome Orc1/Cdc6 functionally complements yeast Cdc6**. In other eukaryotes, Cdc6 has strong sequence similarity with Orc1 (5, 38). Therefore, there are not distinct subdomains within Orc1/Cdc6 that are related to either Orc1 or Cdc6. Instead, Orc1/Cdc6 from trypanosomes is homologous to Orc1/Cdc6.
and also to Cdc6 from other eukaryotes. To confirm that Orc1/Cdc6 is a component of the prereplication machinery in trypanosomes, and moreover, to check whether these proteins could function as Orc1 or as Cdc6 in eukaryotes, we performed a complementation assay in thermosensitive yeast mutants (see Materials and Methods). Strain OAy741 is a cdc6 mutant that is not functional at 37°C, and OAy661 is an orc1 mutant that is not functional at 37°C. Both strains grew at the permissive temperature (23°C) and were not able to grow at the nonpermissive temperature (37°C). When transfected with the empty vector, the mutants grew at 23°C but could not grow at 37°C. When the OAy741 mutant was transfected with the TcOrc1/Cdc6 or TbOrc1/Cdc6 gene, it was able to grow at 37°C, while the OAy661 mutant was not (Fig. 3A and C). Immunoblotting assays using anti-TcOrc1/Cdc6 against transfected yeast cell extracts showed that both strains expressed TcOrc1/Cdc6 (Fig. 3B) or TbOrc1/Cdc6 (Fig. 3D). These results indicate that both TcOrc1/Cdc6 and TbOrc1/Cdc6 are able to complement the yeast cdc6 mutant, but not the orc1 mutant.

**Induction of Orc1/Cdc6 silencing by RNAi results in enucleated cells.** In order to infer that Orc1/Cdc6 is indeed a component of the prereplication machinery in trypanosomes, we performed a functional analysis inducting Orc1/Cdc6 silencing by RNAi. *T. brucei* procyclic forms were transfected with the p2T7-177 vector containing a TbOrc1/Cdc6 fragment. Six days after RNAi induction, the expression of TbOrc1/Cdc6 diminished (Fig. 4A), as well as the growth of RNAi-induced cells (Fig. 4B). DAPI staining of the RNAi-induced cells showed the presence of zoid cells—cells with no nucleus and one kinetoplast (Fig. 4C). Enucleated cells appeared after 4 days of RNAi induction (8% of the culture), increasing until day 7, reaching 20% of the culture. Concomitant with the increment of zoid cells, RNAi induction reduced the number of cells with one nucleus and one kinetoplast (Fig. 4D). To demonstrate that zoid cells are a consequence of nonduplication of nuclear DNA, generating cells that are ready to divide the kineplast, but not the nucleus, we analyzed RNAi-induced cells by FACS analysis. In fact, 7 days after RNAi induction, we could observe cells with a fluorescence intensity lower than the equivalent for G1 cells, which might be cells containing no nucleus and just the kinetoplast. In addition, this culture presents a small amount of G2/M cells (Fig. 4E), indicating that DNA replication might be abolished in these cells. These results are strong evidence that Orc1/Cdc6 is in fact involved in the firing of replication origins in trypanosome cells.

**Expression, localization, and chromatin interaction of the trypanosome Orc1/Cdc6 during the entire cell cycle.** Since we could not find homologues of Cdt1 (a protein involved in the control of DNA rereplication) in the *T. brucei* or *T. cruzi* databases, even when nonannotated sequences were searched,
we investigated whether the restriction of replication to S phase could be via Orc1/Cdc6. Therefore, TcOr1/Cdc6 and TbOr1/Cdc6 expression and their cellular localization were examined during the cell cycle. To validate the anti-TcOr1/Cdc6/Cdc6 specificity in immunofluorescence assay, we incubated epimastigotes from T. cruzi cells in the presence or absence of the recombinant protein rTcOr1/Cdc6. The anti-TcOr1/Cdc6/Cdc6 labeled the nuclei of epimastigotes, and this signal was lost when anti-TcOr1/Cdc6 was first incubated with rTcOr1/Cdc6 labeled the nuclei of epimastigotes, and this signal was lost when anti-TcOr1/Cdc6 was first incubated with rTcOr1/Cdc6 (Fig. 5A).

Immunofluorescence assays of exponentially growing T. cruzi epimastigotes and T. brucei procyclic forms revealed that TcOr1/Cdc6 and TbOr1/Cdc6 are expressed in all cells and are localized in the nucleus in 100% of cells. In T. cruzi and T. brucei, the cell cycle stages can be detected by a morphological analysis that includes counts of flagella, nuclei, and kinetoplasts, a structure that contains the mitochondrial genomic material. T. cruzi cells with one nucleus, one kinetoplast, and one flagellum (1N1k1F) are in the G1 or S phase of the cell cycle. Cells with one nucleus, one kinetoplast, and two flagella (1N1k2F) are in G2 phase. Cells with one nucleus, two kinetoplasts, and two flagella (1N2k2F) are in cytokinesis, and cells with two nuclei, two kinetoplasts, and two flagella (2N2k2F) are cells in cytokinesis (top row in Fig. 5B) (18). When these morphological stages were examined using a monoclonal antibody that labels the flagellum (MABAC) and DAPI staining, which labels the nucleus and kinetoplast, cells in G1/S (1N1k1F), G2 (1N1k2F), mitosis (1N2k2F), and cytokinesis (2N2k2F) showed labeling similar to that for TcOr1/Cdc6 in the nuclear space (Fig. 5B).

The morphological alterations that occur during the cell cycle in T. brucei procyclic forms are not identical to those in T. cruzi. Procyclic forms with just one nucleus, one kinetoplast, and one flagellum (1N1k1F) are in G1 phase. Cells with one nucleus, one kinetoplast, an old flagellum, and a small new flagellum (1N1k2F) are in S phase. Cells with one nucleus, two kinetoplasts, and two flagella (1N2k2F) are in G2 phase, and cells with two nuclei, two kinetoplasts, and two flagella (2N2k2F) are cells in cytokinesis (top row in Fig. 5C) (55). As observed for T. cruzi, all T. brucei cell cycle stages present TbOr1/Cdc6 in the nuclear space (Fig. 5C).

Since TcOr1/Cdc6 and TbOr1/Cdc6 are located in the nucleus during the entire cell cycle and since it has been observed sometimes in mammalian cells that Orc1 is released from the chromatin during S phase (33), we asked whether the TcOr1/Cdc6- or TbOr1/Cdc6-chromatin interaction would change during the cell cycle. Thus, we treated cells with detergent prior to fixation in order to extract proteins not associated with the chromatin. After detergent treatment, the cells were fixed and labeled with anti-TcOr1/Cdc6. As shown in Fig. 6, TcOr1/Cdc6 and TbOr1/Cdc6 were detected in T. cruzi cells (Fig. 6A) and T. brucei cells (Fig. 6B) in different stages of the cell cycle after detergent extraction, indicating that they always remained associated with the chromatin during the cell cycle. For a control, cells were incubated with an antibody that recognizes the soluble heat shock protein 70, and this protein was extracted after detergent treatment (Fig. 6A, panel B, and 6B, panel B). To demonstrate that TcOr1/Cdc6 and TbOr1/Cdc6 are in fact bound to chromatin and not associated with other insoluble structures, cells were treated with DNase after detergent extraction and prior to fixation. In both trypanosomes, the Orc1/Cdc6 protein was released by DNase treatment. In these cases, histone H4, mainly associated with the DNA, was also released from cells after DNase treatment (panels C in Fig. 6A and B). The interaction of TcOr1/Cdc6 and TbOr1/Cdc6 to chromatin was also demonstrated by differential extraction assay followed by Western blotting analysis (Fig. 6C and D), where most of Orc1/Cdc6 proteins are extracted only after DNase treatment.

**DISCUSSION**

Here we present the results of functional analyses of T. cruzi and T. brucei Orc1/Cdc6. These proteins are annotated as Orc1 in the genomic databases of both organisms, but due to the
similarity of Orc1 to Cdc6, we named them Orc1/Cdc6. A single-protein homologue to both Orc1 and Cdc6 is also found in Archaea. Orc1/Cdc6 from Archaea is a member of the AAA+/H11001 ATPase superfamily and is a component of the prereplication machinery that recognizes the replication origin and recruits the MCM complex (49). Searching for specific domains in the trypanosome Orc1/Cdc6, we found that both TcOrc1/Cdc6 and TbOrc1/Cdc6 present an ATP/GTP binding region and residues possibly involved in ATP hydrolysis. Both the T. cruzi and T. brucei proteins are able to hydrolyze ATP. This ATPase activity is in agreement with being a member of the prereplication machinery, whose components from other eukaryotes use ATPase activity to assemble the machinery as well as to control the specificity of origin replication recognition. In fact, the TcOrc1/Cdc6 and TbOrc1/Cdc6 ATPase activities increase in the presence of nonspecific DNA. Analysis of the secondary structure of trypanosome Orc1/Cdc6 also showed the presence of a winged helix domain, the DNA binding domain. However, while the archaeal *A. pernix* Orc1 contains eight positive amino acids in this domain, trypanosomes present three positive amino acids. Therefore, either the trypanosome Orc1/Cdc6-DNA interaction is weaker than the archaeal Orc1/Cdc6-DNA interaction, or it is mediated through other interactions or domains. Since in Archaea, the AAA+ domain also interacts with the DNA (49), it is possible that in trypanosomes, the AAA+ domain is also involved in

FIG. 4. TbOrc1/Cdc6 silencing generates enucleated cells. Procyclic forms of T. brucei 29-13 cell line were transfected by electroporation with p2T7-177 vector containing a fragment of TbOrc1/Cdc6. The synthesis of double-stranded RNA in stable transfected cells was induced by the addition of tetracycline on day zero. Over a period of 8 days, control cells (control) or cells incubated with tetracycline (RNAi induction) were collected and analyzed. (A) The cells were boiled in the SDS-PAGE loading buffer, and the supernatants were analyzed by SDS-PAGE. Membranes were stained with Ponceau and then subjected to Western blotting using either anti-rTcOrc1/Cdc6 antibody or immune serum against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). D5, day 5. (B) The number of cells in the culture was determined. The values are means ± standard deviations (error bars) from three independent experiments. The arrow pointing down indicates when the culture was diluted during the experiment. (C) Seven days after the addition of tetracycline, the cells were harvested by centrifugation, immobilized on glass slides, fixed in 2% formaldehyde, and stained with DAPI. The nucleus (N) and kinetoplast (k) are indicated. The white arrow points to an enucleated cell. (D) The number of nuclei and kinetoplasts in cells stained with DAPI were determined. Eighty cells of each sample were analyzed. The graph shows representative data of three independent experiments. Cells with one nucleus and one kinetoplast (1N1k) (diamonds), cells with one nucleus and two kinetoplasts (squares), cells with two nuclei and two kinetoplasts (2N2k) (triangles), and cells with no nucleus and one kinetoplast (0N1k) (crosses) are indicated. The black arrow pointing down shows the peak of cells containing less DNA than cells in G1.
this interaction. In any case, trypanosome Orc/Cdc6 binds DNA, because the recombinant Orc/Cdc6 from *T. cruzi* binds a simulated bubble replication sequence in gel-shift assay (data not shown). In addition, the ATPase experiment showing that the Orc1/Cdc6 ATPase activity increases in the presence of nonspecific DNA reinforces the data that Orc1/Cdc6 is able to interact with DNA.

Even though the Orc1/Cdc6 from trypanosomes is homologous to yeast Orc1 and Cdc6, complementation assays have shown that TcOrc1/Cdc6 and TbOrc1/Cdc6 replace yeast Cdc6, but not yeast Orc1. Yeast Orc1 has to recognize well-defined ARS (autonomously replicating sequence) elements where the prereplication machinery assembles. This result suggests that trypanosome Orc1/Cdc6 might not be able to recognize the same sequence. It suggests, therefore, that trypanosome Orc1/Cdc6 needs to bind a specific sequence, which is not present in yeast, to license replication origins. Alternatively, the smaller size of trypanosome Orc1/Cdc6 compared to yeast Orc1 (about 100 kDa) could prevent the assembly of trypanosome Orc1/Cdc6 in the yeast ORC complex. On this point, the induction of Orc1/Cdc6 silencing by RNAi in *T. brucei* results in enucleated cells. Taken together, these data show that Orc1/Cdc6 is a component of the prereplication machinery in trypanosomes.

It has already been shown in another trypanosomatid, *Leishmania major*, that Orc1/Cdc6 fused to green fluorescent protein localizes in the nuclear space (31). Here, using polyclonal antibodies that recognize *T. cruzi* and *T. brucei* Orc1/Cdc6, we found that Orc1/Cdc6 is localized in the nuclear space during the entire cell cycle of both the *T. cruzi* epimastigote proliferative form and the *T. brucei* procyclic form. In addition, we provide evidence that the trypanosome Orc1/Cdc6-DNA inter-
FIG. 6. TcOrc1/Cdc6 and TbOrc1/Cdc6 remain bound to chromatin during the entire cell cycle. (A and B) Exponentially growing cultures of *T. cruzi* epimastigote forms (A) and *T. brucei* procyclic forms (B) were harvested by centrifugation, immobilized on glass slides, and sequentially treated to extract soluble proteins (extraction); where indicated, they were also incubated with DNase and then fixed with 2% formaldehyde. For a control, cells on glass slides were fixed in 2% formaldehyde and permeabilized. Cells were incubated with anti-TcOrc1/Cdc6 antibody (panel A), anti-hsp70 (panel B), or anti-histone H4 (panel C) together with MAbAC to label the flagellum and DAPI staining. The nucleus (N), kinetoplast (k), and the new flagellum (f) are indicated. Cells in cytokinesis (C) are indicated. Bars, 1 μm (A) and 4 μm (B). (C and D) The pellets of epimastigote forms from *T. cruzi* (C) and procyclic forms from *T. brucei* (D) were extracted using detergent-containing buffer. After extraction, the cells were subjected to centrifugation, and the supernatants were saved as soluble proteins (soluble fraction 1). The pellets were then extracted again using the same detergent-containing buffer. After extraction, the cells were subjected to centrifugation, and the supernatants were saved as soluble proteins (soluble fraction 2). The pellets were then digested with DNase, samples were centrifuged again, and the supernatant was saved as DNase released proteins (DNase released 1). The pellets were digested with DNase again, the samples were centrifuged, and the supernatants were saved as released proteins (DNase released 2). Total proteins were also analyzed (total extract). Samples were analyzed by SDS-PAGE, followed by Western blotting using anti-rTcOrc1/Cdc6 serum. For controls, samples were also analyzed by SDS-PAGE, followed by Western blot using anti-hsp70 or anti-histone H4.
action is maintained throughout the cell cycle. Therefore, we speculate that in these organisms the restriction of replication at S phase is not due to a different Orc1/Cdc6 localization or to an absence of the Orc1/Cdc6-DNA interaction. The restriction of replication to S phase might be due to posttranslational modifications in Orc1/Cdc6, changes in MCM expression and/or localization during the cell cycle, or the actions of other still-unidentified proteins. These questions are currently under investigation.

The presence of just one protein working as Orc1 and Cdc6 place trypanosomes closer to eubacteria and archaeeae, which possess a single protein to recognize the origins of DNA replication and assemble the DNA helicase on chromatin prior to the initiation of DNA replication. It has been speculated that eukaryotes have six essential proteins to perform the same function, because the temporal regulation of the initiation of DNA replication throughout S phase requires more coordination with the cell cycle progression than in bacterial and archaeeal cells (48). However, our data show that trypanosomes, which are eukaryotes that must control replication and ensure correct segregation of all chromosomes, contain just one protein to assemble the prereplication machinery on DNA. Trypanosomes should be regarded as early divergent eukaryotes. The way that these organisms control rereplication might be different and less complex than eukaryotes containing six ORCs and one Cdc6. Therefore, trypanosomes could be viewed as excellent models to understand the transition from a cell with one chromosome, no nucleus, and one protein licensing the replication origin to a cell with many chromosomes, a nucleus, and a multiprotein complex establishing multiple replication origins.

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