Stage-specific Differences in Cell Cycle Control in Trypanosoma brucei Revealed by RNA Interference of a Mitotic Cyclin

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African trypanosomes have a tightly coordinated cell cycle to effect efficient segregation of their single organelles, the nucleus, flagellum, and kinetoplast. To investigate cell cycle control in trypanosomes, a mitotic cyclin gene (CYC6) has been identified in Trypanosoma brucei. We show that CYC6 forms an active kinase complex with CRK3, the trypanosome CDK1 homologue, in vivo. Using RNA interference, we demonstrate that absence of CYC6 mRNA results in a mitotic block and growth arrest in both the insect procyclic and mammalian bloodstream forms. In the procyclic form, CYC6 RNA interference generates anucleate cells with a single kinetoplast, whereas in bloodstream form trypanosomes, cells with one nucleus and multiple kinetoplasts are observed. Fluorescence-activated cell sorting analysis shows that bloodstream but not procyclic trypanosomes are able to reinitiate nuclear S phase in the absence of mitosis. Taken together, these data show that procyclic trypanosomes can undergo cytokinesis without completion of mitosis, whereas a mitotic block in bloodstream form trypanosomes inhibits cytokinesis but not kinetoplast replication and segregation nor an additional round of nuclear DNA synthesis. This indicates that there are fundamental differences in cell cycle controls between life cycle forms of T. brucei and that key cell cycle checkpoints present in higher eukaryotes are absent from trypanosomes.

The African trypanosome, Trypanosoma brucei, is a unicellular parasitic protozoan that causes sleeping sickness in humans. T. brucei has a complex biphasic life cycle with different developmental forms playing specific roles within each of two hosts (1). The procyclic form in the tsetse fly gut and the long slender form in the mammalian bloodstream establish infection in each host, whereas the metacyclic form in the tsetse salivary glands and the short stumpy form in the mammalian bloodstream are arrested in G0/G1 phase of the cell cycle and are preadapted to life in the new host. Upon transmission, concomitant with differentiation to the new developmental form, the cell cycle arrest is released. Thus, there is an integral link between the cell cycle and parasite differentiation during the life cycle of T. brucei (2). In addition, there is a requirement for the trypanosome to replicate and segregate its single organelles (the nucleus, the kinetoplast (which contains the DNA of the single mitochondrion), flagellum, and basal body) (3, 4), which implies added complexity in cell cycle control not present in relatively simple eukaryotes such as yeast.

Ultrastructural studies of the procyclic form have described a number of markers of cell cycle position and identified discrete phases within the trypanosome cell cycle (3). During G1 phase, the basal body matures. Daughter flagellum outgrowth follows, and new basal bodies for each mature basal body are formed. Kinetoplast S phase is much shorter than nuclear S phase and commences just prior to nuclear S phase (4), suggesting that (as yet unidentified) interorganellar control mechanisms may coordinate DNA synthesis. Early in G2 phase, the replicated kinetoplast segregates, and this is followed by nuclear mitosis and finally cytokinesis. It has been postulated that entry to cytokinesis may be more dependent on kinetoplast division and segregation than on mitosis (5).

Tightly controlled regulation of DNA replication and chromosome segregation is essential for maintenance of genome stability. Eukaryotes have therefore evolved a complex ordered series of checkpoints in order to bring about accurate DNA replication, mitosis, and cytokinesis. Most checkpoints and cell cycle control proteins are highly conserved from yeast to humans. However, at least one cell cycle checkpoint present in mammalian cells has been suggested to be absent from trypanosomes, since treatment of procyclic trypanosomes with the anti-microtubule agent rhizoxin results in cytokinesis occurring in the absence of mitosis (5, 6). In contrast, treatment of procyclic trypanosomes with aphidicolin prevented cells from entering mitosis, indicating that a mitotic entry checkpoint is present (5). It also remains possible that additional checkpoints may operate to cope with requirements specific to trypanosomes (e.g. in order to coordinate the replication and segregation of the kinetoplast with that of the nucleus).

The involvement of cyclin-dependent kinases in the control of the cell cycle is well established in yeast and higher eukaryotes (7, 8). In T. brucei, a number of putative CDKs (known as Cdc2-related kinases (CRKs)) and cyclins have been described (1, 9, 10). The cyclins can be grouped into different classes according to sequence homology to other known cyclins. CYC2, CYC4, CYC5, and CYC7 exhibit homology to the PREG1/PHO80 class of cyclins (1, 10) and may play roles in nutrient sensing; CYC3, CYC6, and CYC8 share homology with mitotic

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cyclins; and CYC9 is a cyclin C homologue and thus may play a role in transcriptional regulation in *T. brucei* (1). Recently, CRK3 was shown to form an active kinase complex with CYC2 in *in vivo*, the first example of a trypanosome CDK-cyclin complex; however, the role of this complex in *in vivo* is not known (10). Studies on *T. brucei* CRK3 and its orthologue from *Leishmania* suggest that CRK3 is a functional trypanosomatid CDK1 (1). By a combination of yeast two-hybrid analysis and co-immunoprecipitations with histone H1 kinase assays, we extend previous studies to show that CRK3 also forms an active kinase complex with CYC6 in *in vivo*.

We demonstrate using RNA interference (RNAi) that CYC6 is essential for mitosis in the African trypanosome, but that a mitotic block produces different phenotypes in the procyclic and bloodstream life cycle stages. In the procyclic form, anucleate cells with a single kinetoplast (termed zoids) (5, 6) are generated, whereas in bloodstream form trypanosomes, cells with a single nucleus but multiple kinetoplasts are observed. Bloodstream form but not procyclic trypanosomes reinitiate nuclear S phase in the absence of mitosis. From these data, we conclude that procyclic trypanosomes can undergo cytokinesis without completion of mitosis, but inhibiting mitosis in bloodstream form trypanosomes prevents cytokinesis but neither kinetoplast replication and segregation nor an additional round of nuclear DNA synthesis.

**EXPERIMENTAL PROCEDURES**

**Recombinant DNA Techniques—**Standard DNA techniques were performed as described (11). Transformation of yeast was achieved using the lithium acetate method (12). Plasmid isolation from *Escherichia coli* and DNA extraction from agrose gels used Qiagen and Qiaex kits from Qiaegen, respectively. DNA sequencing was performed by the DNA sequencing service (University of Glasgow) using an ABI automatic sequencer.

**Cloning CYC2 and TbCKS1—**The *T. brucei* genome databases (available on the World Wide Web at www.tigr.org/tdb/mdb/tbbdb and www.sanger.ac.uk/Projects/T_bruc) were searched using the sequences of known cyclins to identify potential *T. brucei* homologues. Using sequence information derived from these databases, CYC2 was cloned using a PCR-based approach. The sequences of CYC6 have been submitted to EMBL under accession numbers AJ496539 and AJ496540. The genome databases were also searched for the *T. brucei* homologue of *SUC1/CKS1* using the leishmanial CKS1 sequence (13). PCR with oligonucleotides OL861 and OL862 (Table I) was used to amplify the *TbCKS1* sequence fromEATRO 795 genomic DNA. The gene was cloned, sequenced, and submitted to EMBL under accession number AJ496538.

BLAST searches were carried out using the search engine at NCBI (available on the World Wide Web at www.ncbi.nlm.nih.gov/BLAST), which also incorporated a conserved protein domain search.

**Yeast Two-hybrid Assay—**The Hybrid Hunter system (Invitrogen) was used. Plasmids pGL176 (LexA:CRK3) and pGL177 (LexA:CRK3) have been described previously (10). CRK4 (accession number AJ413200) was cloned into the SacI/SalI sites of pHyB/LexZeo following PCR with oligonucleotides OL375 and OL376, generating plasmid pGL395. The open reading frame of CRK3 was previously cloned into pHyB/LexZeo, a 1.6-kb EcoRI-Xhol fragment from pGL220 was subcloned into the EcoRI-Xhol sites of pHyB/LexZeo, generating pGL412. This fragment contained a 5′-truncated CRK5 gene, since CRK5 contains an internal EcoRI site. PCR with oligonucleotides OL490 and OL491 was used to amplify the remainder of the gene and clone it into the EcoRI site of pGL412, generating pGL442 (LexA: CRK5). CRK6 (accession number AJ505556) was cloned into the EcoRI/XhoI sites of pHyB/LexZeo following PCR with oligonucleotides OL849 and OL850, generating plasmid pGL627.

A fragment of CYC6 containing the cyclin box homology region was amplified by PCR using oligonucleotides OL885 and OL896 and cloned into BamHI/HindIII-cut pYESStrp to generate pGL680 (B42:CYC6). *TbCKS1* was cloned into the HindIII and XhoI sites of pYESStrp following PCR with oligonucleotides OL861 and OL862, generating plasmid pGL656 (B42:CKS1). Plasmid inserts were sequenced to show that they were in-frame with either LexA or B42 and contained no mutations introduced by PCR.

Bait and prey plasmids were transformed separately into *Saccharomyces cerevisiae* strain L-40, and β-galactosidase filter lift assays (as described by the manufacturer) were performed to confirm that no plasmid alone could activate transcription of the reporter gene lacZ. L-40 strains expressing appropriate pairs of plasmids were then generated, and further β-galactosidase assays were performed to assay for protein-protein interactions. Control plasmids expressing LexA:Fos, B42-Jun, and B42: lamin (Invitrogen) were also transformed into L-40 in the appropriate combinations to provide positive and negative controls for the assays. Expression of fusion proteins was confirmed by Western blotting of cell lysates with antibodies against either LexA or the V5 epitope, which is fused to the B42 domain, according to the manufacturer’s protocol.

**Antibodies and Immunoblotting—**Antiserum specific for *T. brucei* CRK3 was generated by immunizing a sheep with recombinant CRK3 that was tagged with 6 histidines at the C-terminal end, generating antibody AB24. The mouse monoclonal antibody B2B (15) was used as the anti-TY antibody. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Western blots were performed as described previously (13) with either a 1:20 dilution of anti-TY antibody or a 1:2000 dilution of AB24 followed by a 1:20,000

### Table I

| Oligo     | Gene       | Sequence 5′ → 3′ |
|-----------|------------|-----------------|
| OL171     | CYC2 sense | GCCCATGGCATTATGGTGGACGG |
| OL372     | CRK4 sense | GAGAGCTCTAGTCCAUTGCAAGACTG |
| OL273     | CRK4 antisense | GTCTGGAACAACTACCTGGCAAG |
| OL495     | CRK5 sense | TTCTGGACATCGTACGGTCTTTGG |
| OL491     | CRK5 antisense | CTGGGATCCCGCTATCGGAGTACT |
| OL544     | CYC6 sense | CGGCGCACGCCCTTTGCC |
| OL547     | CYC6 antisense | CCTCGACCTGCGACTCCACAC |
| OL746     | CRK4 5′-flank, sense | TCGAAGCTTATGTCTGGCCGGGACTAC |
| OL827     | PIGO sense | GGATTCCGCGTTCATTCAGGAG |
| OL828     | PIGO antisense | CCGGCGACGCCCTTTGCC |
| OL849     | CRK6 sense | CACCACTTACATACGGAGGTCCTTGG |
| OL850     | CRK6 antisense | GGTTGTCGACGGAGGGGAAAGGCTAGTGGTC |
| OL861     | CKS1 sense | GCGGCTGAGCTCATCTTGGGGTTCTGG |
| OL862     | CKS1 antisense | GAGAGCTTATGTCTGGCCGGGACTAC |
| OL865     | CYC6 sense | CCGGAGGCTTTGAGGAGG |
| OL896     | CYC6 antisense | CCGGAAGCTTTGAGGAGG |
| OL876     | CYC6 5′-flank, sense | CGGCGACGCCCTTTGCC |
| OL941     | CRK6 sense | TACGCGGGAGGCTCTACATACGGAGGTCCTTGG |
| OL952     | CRK6 antisense | TGAGGTCGAAGTGGATCTCTGGTATGAGCTGACC |
| OL953     | CRK6 antisense | CCGGACCTTGATGACCCCAAC |
| OL1155    | CYC6 sense | TACGCGGGAGGCTCTACATACGGAGGTCCTTGG |
| OL1156    | CYC6 antisense | CCGGACCTTGATGACCCCAAC |
| OL1079    | CYC2 sense | CACCACTTACATACGGAGGTCCTTGG |
| OL862     | CKS1 antisense | TGAGGATTTGGCATCTGGAGGAG |
| OL375     | CRK4 sense | CCGGACCTTGATGACCCCAAC |
| OL376     | CRK4 antisense | CCGGACCTTGATGACCCCAAC |
| OL378     | CRK4 5′-flank, sense | CCGGACCTTGATGACCCCAAC |

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2 J. J. Van Hellemond and J. C. Mottram, unpublished results.
of the appropriate horseradish peroxidase-conjugated secondary antibody. The West-Dunm chemiluminescence detection system (Pierce) was used to visualize antigens.

**Transfection of T. brucei**—Culture and transfection of T. brucei was carried out essentially as described previously (16). Where necessary, hygromycin was added at a concentration of 50 μg ml⁻¹ (procyclic cells) or 5 μg ml⁻¹ (bloodstream form cells). G418 at 10 μg ml⁻¹ (procyclic cells) or 2.5 μg ml⁻¹ (bloodstream form cells), zeocin at 10 μg ml⁻¹ (procyclic cells), and phleomycin at 2.5 μg ml⁻¹ (bloodstream form cells).

**Generation of a T. brucei CYC6-ty overexpressing Cell Line**—To generate TY-tagged CYC6, two annealed oligonucleotides OL582 and OL583, which composed the TY epitope flanked by Bpu11102I-compatibili fragments were digested into the unique SpeI site of CYC6. Correct integration of the TY tag was confirmed by sequence analysis. CYC6ty was amplified by PCR using the oligonucleotides OL1155 and OL1156, sequenced to confirm no mutations had been introduced by PCR and ligated into HindIII ApaI-cut pHDS75 (14) to generate pGLO540. Plasmid pGLO540 was linearized by digestion with NotI and transfected into the procyclic cell line EATRO 795 pHD449 (10).

**Overexpression of CYC6ty** was induced in midlog phase cultures by growing cells in medium containing 50 μg ml⁻¹ tetracycline for 48 h. Cells were harvested by centrifugation, washed in phosphate-buffered saline, and either snap-frozen in a dry ice/ethanol bath (for immunoprecipitations and kinase assays) or resuspended in Laemmli buffer (for SDS-PAGE and Western blot analysis). The consensus sequence for the destruction box in CYC6-ty resulted in a cell line containing a partial gene sequence displaying high similarity to mitotic A and B type cyclins. Using PCR, we were able to clone and sequence the remainder of the gene (Fig. 1A), which we designated CYC6. Sequence analysis of independent PCRs indicated that there are two alleles of CYC6. The first allele (CYC6-1) encodes a 436-amino acid protein with a leucine at position 309. The second allele (CYC6-2) contains an insertion of a G at position 827 of the open reading frame. Evidence for the presence of two alleles rather than two genes encoding CYC6-1 and CYC6-2 was provided by BLAST searches of CYC6-1 revealed that it shares highest homology to both N- and C-terminal cyclin fold domains, showing that CYC6 has the structural characteristics of a mitotic cyclin. However, the presence of alleles encoding two distinct proteins is unusual and has not been reported for any other cell cycle-related gene in T. brucei.

**RESULTS**

**Identification of a T. brucei Mitotic Cyclin**—We searched the T. brucei genome databases and identified a sheared DNA clone containing a partial gene sequence displaying high similarity to mitotic A and B type cyclins. Using PCR, we were able to clone and sequence the remainder of the gene (Fig. 1A), which we designated CYC6. Sequence analysis of independent PCRs indicated that there are two alleles of CYC6. The first allele (CYC6-1) encodes a 436-amino acid protein with a leucine at position 309. The second allele (CYC6-2) contains an insertion of a G at position 827 of the open reading frame, resulting in a frameshift and a shorter protein of 426 amino acids (Fig. 1B).

CYC6-2 also has a valine residue at position 309 in place of the leucine. Evidence for the presence of two alleles rather than two genes encoding CYC6-1 and CYC6-2 was provided by BLAST searches of CYC6-1 revealed that it shares highest homology to both N- and C-terminal cyclin fold domains, (accession numbers gn1CDD9502 and gn1CDD6896), thus showing that CYC6 has the structural characteristics of a cyclin. Further feature characteristic of mitotic cyclins is the presence of a 9-amino acid destruction box, frequently present at the N-terminal end of the protein, which targets the cyclin for ubiquitin-mediated proteolysis via the anaphase-promoting complex at the end of mitosis (21). The consensus sequence for the destruction box in
organisms studied to date is RXALGXIIXN. In the absence of data on destruction box sequences in T. brucei, the best candidate is at positions 3–11 (Fig. 1B), but it diverges from the consensus sequence and, most notably, lacks the conserved arginine at position 1 (Fig. 1D).

Evidence that CYC6 can function as a cyclin came from the observation that the cyclin box homology region of CYC6 could complement the S. cerevisiae G1 cyclin conditional mutant, DL-1 (22) (data not shown). We were therefore interested to discover its kinase partner in T. brucei. The cyclin box homology region of CYC6 was tested in a two-hybrid interaction screen with CRK1 and CRK3-CRK6. We were unable to test for an interaction with CRK2, since LexA::CRK2 was autoactivatory in the absence of prey. CYC6 expressed in the host strain L-40 resulted in negligible /H9252-galactosidase activity, whereas a positive reaction was detected for CYC6 with CRK3 (Fig. 2) but not CRK1, CRK4, CRK5, or CRK6 (not shown). To confirm this interaction in vivo, a T. brucei cell line that expresses a tetracycline-inducible TY1-epitope-tagged CYC6 gene was generated (Fig. 3A).

CYC6ty was immunoprecipitated from cell lysates with an anti-TY monoclonal antibody and demonstrated to be associated with a histone H1 kinase activity (Fig. 3B). Preincubation of the antibody with TY peptide abrogated the activity. To determine whether CYC6ty interacts with CRK3 in vivo, CYC6ty immunoprecipitates were analyzed by Western blotting.

**Fig. 1.** Identification and sequence analysis of T. brucei CYC6. A, genetic organization of CYC6. The open reading frame for CYC6 (bases 1402–2709) is shown as a hatched arrow. The 5′-flanking sequence (bases 1–1402) and the 3′-flanking sequence (bases 2710–4412) are shown as gray lines. Key restriction sites are shown, with the position relative to the start of the 5′-flank given in brackets. The positions of oligonucleotides used in this study are shown, with the directions indicated by small arrows. The sequence used for the RNAi construct pGL622 and the probe for the Southern blot (Fig. 1C) are indicated. B, alignment of T. brucei CYC6 with other mitotic cyclins. Protein sequences of the two alleles of CYC6 from T. brucei, CYC6-1 and CYC6-2, with the cyclin box homology regions of cyclin 1 from A. majus (AmCYC1), cyclin S13-6 from G. max (GmCYC1), and human cyclins B1 and A2 (HsCYCB1 and HsCYCA2, respectively) are aligned. Identical residues are in white with dark gray shading, whereas conserved residues are in black with light gray shading. The numbers refer to the position of residues within each protein. The black bar above residues 3–11 of the alignment indicates the position of the putative destruction box.

C, Southern blot of EATRO 795 genomic DNA, probed with CYC6. DNA was digested with the following restriction enzymes: EcoRV (lane 1); XhoI (lane 2); EcoRV and XhoI (lane 3); KpnI (lane 4); KpnI and EcoRV (lane 5); and KpnI and XhoI (lane 6). D, alignment of the destruction boxes from the mitotic cyclins shown in B.
We were also able to identify and clone *T. brucei* CKS1, a homologue of leishmanial CKS1 (13) and *S. cerevisiae* SUC1. The trypanosome p12\(^{dsi}\) shares 67\% identity with p12\(^{ksi}\) of *Leishmania mexicana*. In the two-hybrid assay, a positive interaction, albeit weak, was detected for CRK3 and the *T. brucei* CKS1, but not when CKS1 was expressed alone (Fig. 2), indicating that trypanosomone p12\(^{dsi}\) can interact with CRK3. This finding is consistent with the ability of CRK3 to interact with leishmanial p12\(^{ksi}\) (10).

**CYC6 RNAi in Procyclic Trypanosomes**—In order to further investigate the role of CYC6 in *in vivo*, an RNAi approach was used. A fragment (see Fig. 1A) of CYC6 was cloned into vector p2T7ti (18) and transfected into the *T. brucei* procyclic 427 pLew13 pLew29 cell line (17). Two independent clones were selected, and growth curves were generated in the absence or presence of tetracycline, which induces expression of the CYC6 double-stranded RNA (Fig. 4A). Induction of CYC6 RNAi resulted in a growth arrest in procyclic cells ~48–72 h post-induction. No such growth arrest was seen upon tetracycline induction of the RNAi cell line transfected with the p2T7ti construct containing the coding sequence for GFP (Fig. 4A), thus indicating that the growth arrest seen with CYC6 RNAi is not caused by the production of double-stranded RNA per se but is specifically associated with production of CYC6 double-stranded RNA.

To confirm that the growth arrest resulted from a specific down-regulation of CYC6 mRNA, an RT-PCR method was used. This approach was taken, since CYC6 could not be detected reliably by Northern analysis due to low abundance. cDNA generated from mRNA with or without induction was used in multiplex PCRs with primers for PIGO and either CYC6 or CYC2. One primer used to amplify CYC6 binds outside of the region used in the RNAi construct (Fig. 1A); hence, any products obtained should be derived solely from native RNA and not from RNA produced from the RNAi construct. The PCR products were then electrophoresed and subjected to Southern blotting with the relevant probes. *T. brucei* PIGO, a gene involved in lipid anchor biosynthesis,\(^3\) was used as a loading control. The ratio of CYC6 or CYC2 product to PIGO product was assessed on a phosphor imager. Since each independent clone gave essentially the same phenotype, for this and subsequent analyses, only the data for one clone is shown.

Induction of CYC6 RNAi in procyclic cells led to a dramatic decrease in CYC6 mRNA relative to PIGO after 28 h, whereas the CYC2 mRNA levels remained approximately constant (Fig. 4B). This indicates that the effects of the RNAi are due to a specific down-regulation of CYC6 mRNA.

To confirm that knockdown of CYC6 RNAi was associated with a down-regulation of CRK3-CYC6 kinase activity, cell lysates were assayed for p13-binding kinase activity. Following induction of CYC6 RNAi, the p13-binding kinase activity decreased to background levels (Fig. 4C), consistent with the notion that CYC6 RNAi leads to a reduction in CYC6 protein levels, resulting in reduced CRK3-CYC6 kinase activity.

The karyotype distribution of the cell populations was monitored through the time course by using DAPI staining to visualize nuclei and kinetoplasts (Fig. 5). Induction of CYC6 RNAi in procyclic form trypanosomes resulted in an absence of cells progressing through the cell cycle to a two-nuclei, two-kinetoplast (2N2K) karyotype, while at the same time giving rise to significantly more 1N2K and 0N1K (“zoid”) cells (Fig. 5A). Indeed, within the population, isolated cells of the 1N2K karyotype could be seen dividing to give a 1N*1K daughter cell (1N* being where the nucleus has a 4C DNA content; see

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\(^3\) S. G. Lillico and J. C. Mottram, unpublished results.
below) and a zoid (Fig. 5B), suggesting that the absence of mitosis did not prevent cytokinesis.

To measure the DNA content of the cells through the time course, cells were analyzed by FACS (Fig. 6). RNAi of CYC6 resulted in a decrease in the proportion of cells with 2C DNA content, whereas the proportions of cells with either <1C DNA content or 4C DNA content increased. This is in keeping with the observed karyotype of the cells (i.e., an increase in 1N2K cells that have replicated their nuclear DNA (4C content, designated 1N*1K) and an increase in zoids (<1C DNA content)). The absence of cells with DNA contents greater than 4C indicates that, within the population as a whole, 1N*1K daughter cells do not undergo a further round of nuclear S phase.

**CYC6 RNAi in Bloodstream Form Trypanosomes—**To test whether RNAi of CYC6 would give the same phenotype in bloodstream form cells, the same construct was transfected into cell line 427 pLew13 pLew90-6 (17), and two independent clones were selected for further analysis. RNAi of CYC6 in bloodstream form cells resulted in a growth arrest after overnight induction (Fig. 7A). This growth arrest was associated with a specific reduction in CYC6 mRNA as determined by RT-PCR (Fig. 7B). After induction of CYC6 RNAi, a significant
thus indicating that the effects of the RNAi are due to a specific down-regulation of CYC6 mRNA. Assaying the p13-binding kinase activity following induction of RNAi showed a decrease in CRK3-CYC6 activity down to background levels (Fig. 7C).

An analysis of the karyotype distribution in the induced bloodstream form cells revealed a significant increase in 2N2K cells with a coincident decrease in 2N2K cells, together with cells with multiple kinetoplasts (1N3K, 1N4K increasing up to 1N11K) (Fig. 8). In the multikinetoplast cells, the nucleus was usually significantly enlarged and often bilobed. Cell morphology was frequently aberrant. These results suggest that the cells are unable to complete mitosis, but unlike the situation in procyclic cells, cytokinesis does not occur. Absence of cytokinesis does not, however, prevent kinetoplast replication and segregation or outgrowth of a new flagellum.

Since the nuclei in the multikinetoplast cells were significantly enlarged, it seemed possible that the arrested bloodstream form cells were undergoing repeated rounds of nuclear S phase in the absence of mitosis. FACS analysis (Fig. 9) showed over time an increase in cells with a DNA content of greater than 4C, coincident with a decrease in cells with a 2C DNA content. However, unlike the case for the procyclic form, this was accompanied by an increase in cells with DNA contents of greater than 4C, suggesting that the single nucleated, multikinetoplast cells underwent further rounds of nuclear S phase. FACS analysis on the host strain 427 pLew13 pLew90-6 revealed the same profile as the uninduced RNAi cell line (data not shown), confirming that this strain was not tetraploid prior to transfection with the RNAi construct.

**DISCUSSION**

We report the identification and analysis of a novel mitotic cyclin gene CYC6 from *T. brucei*, thus providing the first analysis of a molecule crucial to mitosis in the African trypanosome. We have the classification of CYC6 as a mitotic cyclin on the high degree of sequence similarity to many B-type cyclins, the presence of a sequence motif similar to a destruction box, and its functional complementation of the *S. cerevisiae* G1 cyclin conditional mutant α1-1. This strain has previously been shown to be promiscuous for the type of cyclin that can complement it (12, 22). Further, we have shown that CYC6α1-1 is able to interact with CRK3, the proposed functional Cdc2/CDK1 homologue in kinetoplastids (1, 23), to form an active kinase complex. This is only the second cyclin-kinase complex in African trypanosomes to be identified. CRK3 interacts with the PREG1/PHO80-like (24) trypanosome cyclin CYC2 (10) to form a p12^α1-1-binding complex and with the mitotic cyclin CYC6 to form a p13^α1-1-binding complex. Considering the proposed classifications of the cyclins, it is likely that CRK3 interacts with CYC2 in G1 and has a G1/S-specific function, perhaps operating by sensing cell size or nutritional status, and with CYC6 at G2/M to bring about progression through mitosis. Recent data to support this is the finding that RNAi of CYC2 in the procyclic form of *T. brucei* leads to a G1 cell cycle block.

Finally, using RNAi, we have shown that down-regulation of CYC6 mRNA results in a reproducible mitotic block phenotype in both procyclic and bloodstream form trypanosomes (Table II). Induction of CYC6 RNAi caused a growth arrest after 48–72 h in procyclic cells and after 10–16 h in bloodstream form cells. The differences in rapidity of onset of the phenotype may, at least in part, be accounted for by the differences in generation time of the two life cycle forms (~10–12 h for the procyclic form and 5–6 h for the bloodstream form). Additionally, the fact that procyclic cells were able to undergo cytokinesis following the mitotic block means that the growth arrest

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4 T. C. Hammarton, M. Engstler, and J. C. Mottram, unpublished results.
did not become apparent until one cell cycle later.

In procyclic trypanosomes, knockdown of CYC6 mRNA resulted in an almost total absence of 2N2K cells, showing that cells were unable to undergo nuclear mitosis, although they were able to segregate their kinetoplasts. This indicates that CYC6 is required for nuclear mitosis but not for division of the replicated kinetoplast. FACS analysis confirmed that these cells did not reinitiate nuclear S phase but were able to undergo cytokinesis in the absence of mitosis to give a daughter cell with double nuclear DNA content (1N*1K) and a zoid (0N1K). This phenotype has previously been seen as a result of treating procyclic trypanosomes with the anti-microtubule agent rhizoxin (5, 6). This work confirms, using ablation of a molecule essential for entry into mitosis, that procyclic form trypanosomes lack a cell cycle checkpoint preventing initiation of cytokinesis in the absence of completion of mitosis, which is present in other eukaryotes.

In bloodstream form cells, RNAi knockdown of CYC6 resulted in a significant decrease in the formation of 2N2K cells, consistent with CYC6 being required for mitosis. In many cases, the nuclei of the aberrant bloodstream form cells were significantly enlarged and bilobed, often to such an extent that only a very thin thread of DNA connected the two lobes. In contrast, bilobed nuclei were not seen in the procyclic 1N*2K cells. It is unlikely that these differences are a result of differing levels of penetrance of the RNAi in the two life cycle stages, since the p13-binding kinase activity is reduced to background levels following RNAi in both stages, suggesting similar levels of CYC6 down-regulation. Instead, this difference in phenotype could indicate that CYC6 acts later in mitosis in bloodstream forms than in procyclic forms. In contrast to the situation in

![Fig. 8. Karyotype of bloodstream form cells after CYC6 RNAi.](image)

A, karyotype distribution. Bloodstream form cells (clone P2A) were induced with tetracycline (tet), and the karyotype distribution was monitored over time. Upper graph, filled circles, 1N1K; filled squares, 1N2K; filled triangles, 2N2K; crosses, other karyotypes. Lower graph, crosses, 0N1K; open triangles, 1N3K; open squares, 1N4K. B, images of bloodstream form CYC6 RNAi cells. Bloodstream form cells (clone P2A) were stained with DAPI (from left to right, phase-contrast, DAPI-stained, and overlay). Top, uninduced; bottom, tetracycline-induced (T = 16 h).

![Fig. 9. FACS analysis of bloodstream form CYC6 RNAi cells.](image)

FACS analysis was performed on clone P2A at 18 and 35 h postinduction. Left, uninduced cells; right, tetracycline (tet)-induced cells.

**Table II**

| Phenotype generated by CYC6 RNAi | Procyclic form | Bloodstream form |
|----------------------------------|---------------|-----------------|
| Growth arrest                   | 48–72 h       | 10–16 h         |
| Generation of zoids             | Yes, 30–35% of total cells | No |
| Generation of multikinetoplast cells | No | Yes, mostly 1N3K or 1N4K, but increasing up to 1N11K |
| Cytokinesis in absence of mitosis | Yes | No |
| Kinetoplast segregation and division in absence of mitosis | Yes | Yes |
| Reinitiation of nuclear S phase in absence of mitosis | No | Yes |
| Reinitiation of kinetoplast division in absence of mitosis | No | Yes |
procytokinesis, However, the absence of large numbers of zoids and the presence of cells with multiple kinetoplasts indicate that bloodstream form trypansomes are unable to undergo cytokinesis in the absence of mitosis. The majority of aberrant cells had karyotypes of 1N3K or 1N4K, but cells with one nucleus and up to 11 kinetoplasts were occasionally seen. This may indicate that, unlike the situation in the procyclic form, the mitosis to cytokinesis checkpoint present in other eukaryotes is operational in bloodstream form trypansomes. Alternatively, it can be considered that structural constraints of cell division in the bloodstream form render cytokinesis impossible in the absence of mitosis. In the procyclic form, it was suggested that entry into cytokinesis may be largely dependent on kinetoplast division and segregation (5). However, it is clear that kinetoplast division and segregation is not sufficient for initiation of cytokinesis in bloodstream form cells.

Bloodstream form cells were also able to reinitiate nuclear S phase in the absence of mitosis or cytokinesis, thus providing an explanation for the enlarged nuclei seen by microscopy. These data indicate that in this form, the checkpoint preventing reentry into S phase until cytokinesis and G1 have been completed is either lacking or has been inactivated by the knockdown of CYC6. The latter option would be consistent with the situation in yeast where reentry into S phase is inhibited by high CDK1 activity (25). Recently, RNAi of FLA1 (flagella adhesion glycoprotein gene) (26) and of GPI8, encoding an enzyme involved in attachment of glycosylphosphatidylinositol anchors to proteins (27) in bloodstream form T. brucei, was shown to result in a block in cytokinesis. In these cells, multiple rounds of nuclear mitosis and kinetoplast segregation as well as flagella duplication in the case of GPI8 RNAi occurred. Thus, it seems that in the absence of cytokinesis in bloodstream form cells, there is no mechanism to prevent multiple rounds of reentry into S phase and organelle segregation. Similarly, if cytokinesis is blocked in the procyclic form of T. brucei by RNAi of FLA1 or a-tubulin or in the epimastigote form of T. cruzi by treatment with vinca alkaloids, repeated rounds of mitosis and kinetoplast duplication can occur (26, 28, 29). It remains to be seen whether in these cells this is due to inactivation of CRK3-CYC6 having occurred prior to the block in cytokinesis or whether trypanosomes lack a traditional mitosis to S phase checkpoint altogether. In the latter case, this would imply that reentry into S phase in the same cell cycle is possible until cytokinesis is initiated. In any case, it will be important to bear this in mind when analyzing RNAi cell lines in the future, since proteins uninvolved in the cell cycle, may, by invoking a block in cytokinesis through a cell cycle-independent mechanism, appear to generate a cell cycle phenotype.

One final point on checkpoints is that, at least in the procyclic form, there must be additional controls operating at G1/S to prevent the 1N*1K (4C nuclear DNA content) daughter cells from undergoing a further round of S phase. In mammalian cells, a p53-dependent G1 tetraploidy checkpoint exists to prevent cells that have failed to segregate their chromosomes during the previous mitosis from progressing through G1, through transactivation of the CDK inhibitor p21 (30, 31). It will prove interesting to discover exactly how procytoplastic trypanosomes bring about a similar arrest.

This work shows that there is an absolute requirement for CYC6 for mitosis, and the phenotypes seen with RNAi (Table II) of CYC6 argue against any redundancy of function between the putative mitotic cyclins in T. brucei. It is possible that one of the other mitotic-like cyclins, CYC3 or CYC8, could be involved in later mitotic events, such as exit from mitosis, or segregation of the two daughter nuclei following nuclear division. Further, it is now apparent that kinetoplast division and segregation are not dependent on the G2/M cyclin-kinase complex CRK3-CYC6, raising the possibility that another cyclin-kinase complex, perhaps involving one of the other mitotic-like cyclins, CYC3 or CYC8, is required for this process in the trypanosome cell cycle. Although the cell cycles of the nucleus and kinetoplast occur approximately synchronously, suggesting that they may be linked, they are not interdependent, as shown by the treatment of procyclic cells with okadaic acid, which prevents kinetoplast replication and cytokinesis but not mitosis (32), and in this study where kinetoplast replication can repeatedly occur in the absence of mitosis. The involvement of different cyclin-kinase complexes in the two cell cycles would provide an explanation for these phenomena.

Finally, although the T. brucei cell cycle has features common to other eukaryotes, such as a single CDK interacting with more than one cyclin to carry out different functions, trypanosome-specific features also exist. Replication and division of its mitochondrion appears to be carefully controlled by as yet unidentified molecules that are not required for nuclear division. Structural constraints of cell division may also mean that different and possibly novel cell cycle control mechanisms operate during mitosis and cytokinesis. Indeed, the unusual mechanism for achieving chromosome segregation in the trypanosome, proposed as a lateral stacking model (33), may indicate that unique molecular pathways underlie mitosis in African trypanosomes. Further careful molecular dissections of the events occurring at mitosis and cytokinesis in both bloodstream and procytoplastic forms will be required to fully determine the operational cell cycle checkpoints in trypanosomes and to elucidate how such control mechanisms evolved in the different life cycle stages.

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Stage-specific Differences in Cell Cycle Control in *Trypanosoma brucei* Revealed by RNA Interference of a Mitotic Cyclin

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