

## Import of proteins into the trypanosome nucleus and their distribution at karyokinesis

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Accepted 13 December 1999; published on WWW 14 February 2000

### SUMMARY

In all eukaryotic organisms proteins are targeted to the nucleus via a receptor-mediated mechanism that requires a specific nuclear localization sequence (NLS) in the protein. Little is known about this process in trypanosomatid protozoa that are considered amongst the earliest divergent eukaryotes. We have used the green fluorescent protein (gfp) and  $\beta$ -galactosidase reporters to identify the NLS of two trypanosomal proteins, namely the *Trypanosoma brucei* La protein homologue and histone H2B of *T. cruzi*. A monopartite NLS was demonstrated at the C terminus of the La protein, whereas a bipartite NLS was identified within the first 40 amino acids of histone H2B. Treatment of live trypanosomes with poisons of ATP synthesis resulted in exit of the La NLS-gfp fusion from the nucleus. Interestingly, this fusion protein accumulated at

several discrete sites in the cytoplasm, rather than equilibrating between the nucleus and the cytoplasm. When ATP levels returned to normal, the protein reentered the nucleus, demonstrating that the process was energy dependent. Finally, using fusion proteins that localize to the nucleoplasm or the nucleolus, we identified a subpopulation of mitotic cells in which the chromosomes have segregated but the daughter nuclei remain connected by a thin thread-like structure. We propose that cells containing this structure represent a late stage in nuclear division that can be placed after chromosome segregation, but before completion of karyokinesis.

Key words: Trypanosome, Nuclear protein, NLS, La, H2B

### INTRODUCTION

The nucleus of all eukaryotic cells from protozoa to mammals is delimited by a specialized double membrane, the nuclear envelope that is perforated by nuclear pore complexes (NPC). NPCs are the sites at which free diffusion of molecules smaller than 10 nm in diameter, and regulated trafficking of macromolecules in and out of the nucleus take place. In particular, transport of karyophilic proteins from their site of synthesis in the cytoplasm to their destination in the nucleus has been shown to occur via a number of signal-mediated pathways that share common features (Corbett and Silver, 1997; Nigg, 1997). Briefly, karyophilic proteins are endowed with a nuclear location signal (NLS) that is recognized by a receptor. The protein/receptor complex is actively transported through the NPC and, once in the nucleus, the complex dissociates and the receptor returns to the cytoplasm. There is heterogeneity both at the level of the NLS and of the specific receptors that recognize them. The prototypical NLS, exemplified by the SV40 large T antigen, is a seven amino acid peptide characterized by a high content of basic amino acids. A variation of this basic residue-rich NLS is the bipartite-type NLS originally identified in nucleoplamin and that consists of two clusters of basic amino acids, with a high prevalence of

lysines, separated by ten amino acids. A third type of NLS, termed M9, has been recently identified in the RNA binding protein hnRNPA1 and bears no similarity to the monopartite and bipartite NLS (Siomi and Dreyfuss, 1995). The heterogeneity of the NLS is reflected at the level of the transport receptors the different NLS interact with. A heterodimeric receptor consisting of importin  $\alpha$  and importin  $\beta$  (also termed karyopherin  $\alpha$  and  $\beta$ ) binds to the classical NLS-bearing proteins and brings them to the transport apparatus located at the NPC. Importin  $\alpha$  binds to the NLS, whereas importin  $\beta$  mediates binding to the NPC. In contrast, a single monomeric receptor, termed transportin, binds to M9 and mediates nuclear import of hnRNPA1 (Pollard et al., 1996). Importin  $\beta$  and transportin are distantly related in sequence and are members of a superfamily of proteins that are implicated as nuclear transport receptors. Other essential components involved in nuclear import of most karyophilic proteins are the small Ras-related GTP-ase, Ran/TC4, and its regulatory factors. There is so far only one example of Ran-independent transport, namely the HIV-1 Vpr protein, which seems to gain nuclear access by using a pathway distinct from the basic NLS and M9 pathway (Jenkins et al., 1998).

In addition to the NPC architecture, many components of the nuclear transport apparatus have been conserved throughout

evolution. At present, however, we have little information concerning nuclear protein targeting in early divergent eukaryotes, like the trypanosomatid protozoa. Briefly, it is known that in certain instances the SV40 T-antigen NLS can mediate nuclear import in *T. brucei* (Wirtz et al., 1994; Xiong and Ruben, 1996) and that a Ran/TC4 homologue exists in *T. brucei* (Field et al., 1995). Because of our interest in nuclear events and nuclear biogenesis in *T. brucei*, we investigated the NLS required for nuclear import of two trypanosomal proteins, namely the *T. brucei* La protein homologue and *T. cruzi* histone H2B. The La protein binds to precursors of RNA polymerase III transcripts, and since its discovery almost two decades ago has been proposed to function in a variety of cellular processes. More recently, the study of its role in yeast has demonstrated that La functions as a molecular chaperone for RNA polymerase III transcripts in vivo (Pannone et al., 1998; Yoo and Wolin, 1997). The NLS of the trypanosome La protein was coincident with the last seven amino acids, was similar to the classical monopartite SV40 large T antigen NLS and was sufficient to mediate ATP-dependent transport of *gfp* into the nucleus. In the case of histone H2B, we found that the amino-terminal 40 amino acids contain a bipartite-type NLS, of which nucleoplamin is the prototype. Furthermore, using *gfp* fusion proteins that localized to different components of the nucleus, namely the nucleoplasm, chromosomal DNA and the nucleolus, we visualized mitotic cells. This revealed that after chromosome segregation the two nuclei remain connected by a thin thread-like structure. This structure did not contain detectable DNA, and was also observed by indirect immunofluorescence of fixed cells with anti-La antibodies.

## MATERIALS AND METHODS

### Trypanosomes

Procyclic *T. brucei* rhodesiense Y Tat1.1 cells were used throughout the experiments. Cells were grown and transfected as previously described (Fantoni et al., 1994). Stably transfected cells were grown as populations and were not cloned. For transient expression, cells were electroporated with 100 µg of plasmid DNA and were analyzed for *gfp* expression as early as two hours after transfection.

### Construction of *gfp* expression vectors

The coding region of *gfp* mutant 3 (a generous gift from Dr Stanley Falkow) was amplified by PCR using a forward primer containing a *Hind*III site and an *Nhe*I site immediately after the ATG initiation codon of *gfp* and a reverse primer harboring an *Eco*RI site. After digestion with *Hind*III and *Eco*RI, the PCR product was cloned in the pXS2 expression vector to generate pXSGFPM3FUS.

For expressing *gfp*- $\beta$ -galactosidase fusion proteins, we started with plasmid pLAC (E. Ullu, unpublished results), which consists of the PARP promoter followed by the calmodulin B gene 3' splice site, the 5'UTR and the first 25 amino acids of calmodulin fused in frame with the  $\beta$ -galactosidase coding region. The unique *Bgl*III site at position 25 of the coding region was ligated to an *Nhe*I adapter to generate pLACN<sup>+</sup>. The coding region of *gfp* was amplified from the pXSGFPM3FUS vector by PCR with primers containing *Xba*I sites. The PCR product was inserted at the *Nhe*I site of pLACN<sup>+</sup> to generate pGFP-LAC, which was then used for cloning the various H2B protein fragments.

### Construction of *gfp* fusion proteins

The coding region of the *T. brucei* La protein was amplified by PCR

using as a template clone pTbLa1 (E. Ullu and J. Morehead, unpublished results, GenBank accession no. AF212865) and specific oligonucleotides containing *Nhe*I sites, and cloned at the *Nhe*I site of expression vector pXSGFPM3FUS. Deletion derivatives were similarly produced using specific oligonucleotide primers. LaNLS-*gfp* was constructed by inserting a double-stranded DNA oligonucleotide encoding the NLS at the *Nhe*I site.

The coding region of *T. cruzi* histone H2B was amplified by PCR using as a template genomic DNA and specific oligonucleotide primers as described above for the La protein. The H2B-specific oligonucleotides were designed based on the sequence deposited in GenBank (accession number X60982). Deletion mutants were generated by PCR and mutagenesis to introduce single or multiple amino acids substitutions in the context of H2B40-*gfp* was done using two successive PCR reactions according to standard procedures. All mutations were confirmed by sequence analysis.

The *T. brucei* fibrillar coding region was amplified from plasmid pTbFIB (a generous gift from Stephen Wormsley and Dr Susan Baserga) and inserted in the pXSGFPM3FUS vector as described above.

### Microscopy

For *Gfp* analyses, cells expressing *gfp* were collected by low speed centrifugation, washed in phosphate buffered saline (PBS) and allowed to settle on poly-L-lysine coated slides. Cells were then fixed for 20 minutes on ice in 4% paraformaldehyde-20% fetal bovine serum (FBS). After fixation cells were washed three times in PBS containing 20% FBS, and permeabilized in the same solution containing 0.025% saponin. DNA was stained with 4,6 diamino-2-phenylindole (DAPI) which was added to the mounting medium. *Gfp* fluorescence was visualized by illumination with UV light and a FITC filter using a Nikon microscope equipped with a Cooled Coupled Device (CCD) camera as described (Ngo et al., 1998).

### Immunostaining

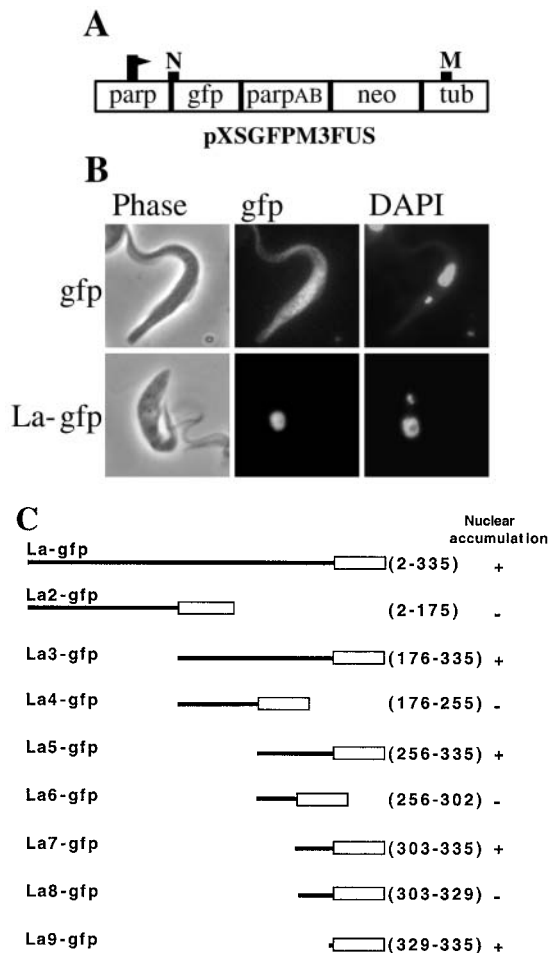
The coding region of the La protein was cloned in the pXpress vector (InVitrogen) in frame with a 6xHis tag. The recombinant protein was purified by chelation chromatography and used as an antigen to immunize rabbits. The polyclonal serum was used for decorating trypanosomes without further purification. For immunofluorescence procyclic cells were deposited on poly-L-lysine coated slides and fixed and permeabilized as described above. After reaction with the primary antibody, cells were washed in PBS and incubated with a goat anti-rabbit rhodamine-conjugated secondary antibody.

## RESULTS

To analyze trypanosomal NLS we chose the La protein homologue of *T. brucei* (S. Wolin, J. Morehead, C. Yoo and E. Ullu, unpublished, GenBank accession no. AF212865) and histone H2B of *T. cruzi* (GenBank accession number X60982), because their corresponding NLS have been characterized in other systems and these proteins are targeted to different nuclear components, namely the nucleoplasm (La) and chromatin (histone H2B).

### The *T. brucei* La protein is targeted to the nucleus via a classical monopartite NLS

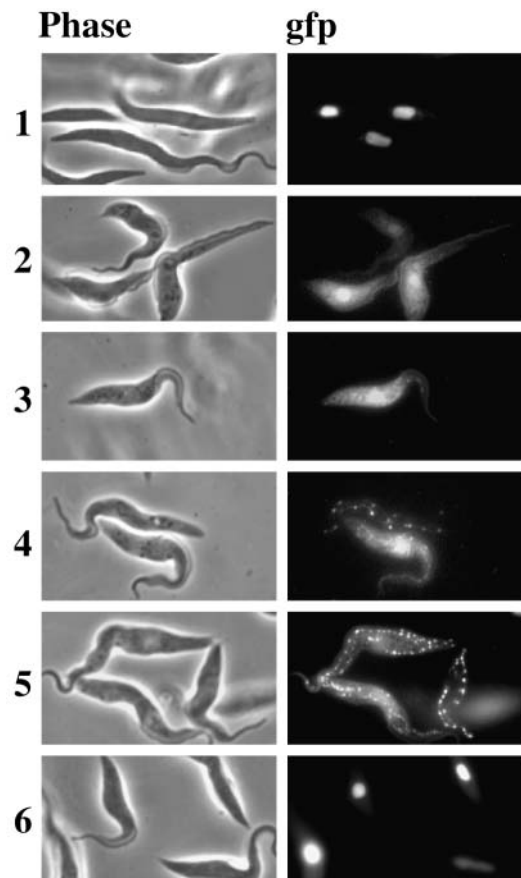
Initial experiments with the *T. brucei* La protein homologue revealed that this protein, as assessed by indirect immunofluorescence, is indeed predominantly localized in the nucleoplasm of procyclic trypanosomes (see Fig. 6C), as it is the case in all other eukaryotes analyzed so far (Yoo and Wolin, 1994). Similarly, the entire La coding region fused to



**Fig. 1.** The last seven amino acids of the La protein encode the NLS. (A) Schematic representation of pXSGFPM3FUS vector. The coding region of *gfp* mutant 3 was inserted in the *T. brucei* expression vector pXS2 (see Materials and Methods for details). In addition to sequences required for propagation in bacteria, the vector contains in a 5' to 3' direction the promoter and RNA processing signals of the procyclin acidic repetitive protein (*parp*), the coding region of *gfp* with an *NheI* site (N) immediately downstream of the *gfp* translation initiation codon, the 3' untranslated region (UTR) and poly(A) site of the PARP A gene, the intergenic region and 3' splice acceptor region and 5'UTR of the PARP B gene (*parpAB*), the neomycin phosphotransferase coding region (*neo*), and the 3'UTR and poly(A) site of the  $\beta$ -tubulin gene followed by the  $\alpha$ -tubulin gene signals required for 3' end formation (*tub*). M, *MluI* site used to linearize the plasmid for insertion into the chromosomal tubulin gene array. (B) Intracellular localization of *gfp* and La-*gfp* in procyclic trypanosomes. pXSGFPM3FUS DNA was transiently expressed in procyclic *T. brucei* cells (panels *gfp*) whereas pLa-*gfp* was linearized at the *MluI* site and, after transfection into cells, a stable cell population was established (panels La-*gfp*). Cells were fixed with paraformaldehyde, permeabilized, stained with DAPI and mounted for microscopy. In the DAPI panels the small dots represent the kinetoplast DNA. (C) Summary of the subcellular localization of La-*gfp* fusion proteins. The coding region of the La protein is indicated by a solid line and that of *gfp* by an open box. Mutations are named on the left and their nuclear accumulation is given on the right. (+), indicates nuclear accumulation in all cells observed; (-), indicates lack of nuclear accumulation in all cells observed. Numbers in parenthesis indicate the amino acid residues fused to *gfp*. The drawing is not to scale.

the amino terminus of *gfp* in the expression vector pXSGFPM3FUS (Fig. 1A) was localized to the nucleus of stably transfected procyclic cells (Fig. 1B, La-*gfp*) and the same localization was observed after transient expression of the same construct (not shown). In particular, La-*gfp* was homogeneously distributed in the nucleoplasm, and seemed less abundant, if at all present, in the nucleolus. In contrast, the fluorescence of *gfp* itself was equally distributed in the cytoplasm and the nucleus of the cell (Fig. 1B, *gfp*), in agreement with the fact that this protein's molecular mass (27 kDa) is well below the 40-60 kDa size limit for passive diffusion of proteins through the NPC.

To identify sequences responsible for nuclear accumulation of the La-*gfp* construct, deletion derivatives were constructed and transfected into trypanosomes (Fig. 1C). The localization of the fluorescence of the corresponding fusion proteins established that nuclear accumulation segregated with the carboxy terminal domain of the protein. Mutant La9-*gfp*, containing only the C-terminal peptide RGHKRSRE (positions



**Fig. 2.** Intracellular localization of La9-*gfp* fluorescence before and after treatment with metabolic inhibitors. A stable cell population expressing La9-*gfp* was incubated for various periods of time with 10 mM sodium azide and 10 mM 2-deoxy-D-glucose at 28°C. Live cells were mounted on microscope slides and *gfp* fluorescence was detected by illumination with UV light and an FITC filter. Panel 1, cells before the addition of the inhibitors; panels 2-5, cells after 5, 10, 20 and 40 minutes of incubation in the presence of the inhibitors; panel 6, cells were washed free of the inhibitors and allowed to recover for 15 minutes at 28°C in the presence of 20% fetal bovine serum.

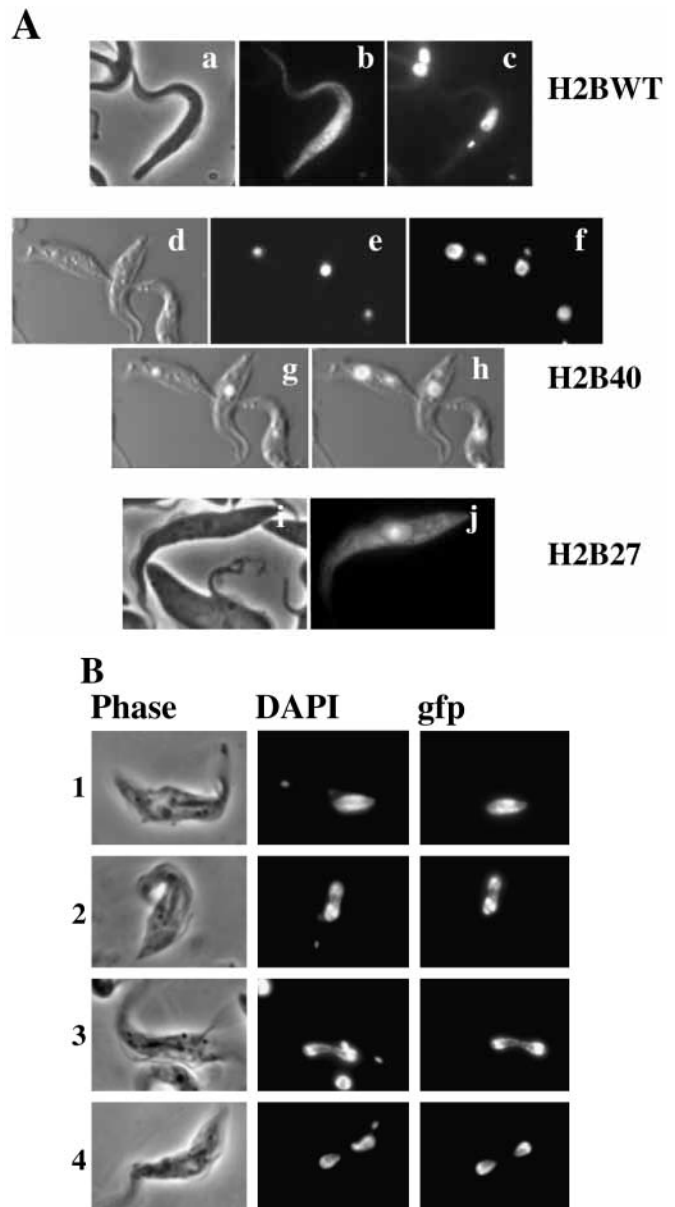
329-335), represented the smallest fragment of the *T. brucei* La protein that produced nuclear accumulation of gfp.

The size of the La9-gfp protein (28 kDa) raised the question whether nuclear accumulation resulted from either or a combination of two possible mechanisms: active nuclear transport of La9-gfp mediated by the La NLS, or passive diffusion of La9-gfp through the NPC and retention in the nucleus by binding to a nuclear component. To distinguish between these two possibilities, we simultaneously incubated trypanosomes stably expressing La9-gfp with two metabolic inhibitors, namely 2-deoxy-D-glucose and sodium azide. Similar studies in yeast have shown that this treatment depletes ATP stores in vivo and induces equilibration of NLS-gfp between the nucleus and the cytoplasm (Shulga et al., 1996). Fig. 2 shows a gallery of images from an uncloned population of La9-gfp cells before (panel 1) and after (panels 2-5) addition of the metabolic inhibitors. 5 to 10 minutes after addition of the inhibitors, the cells became paralyzed and a proportion of gfp fluorescence equilibrated between the nucleus and the cytoplasm (panels 2 and 3). A second phenomenon partially overlapped in time with the exit of gfp from the nucleus, namely the appearance in the cytoplasm of clusters of gfp fluorescence mainly localized to the cell periphery. This phenomenon did not appear in all cells at the same time, as it can be appreciated in panel 4. However, after about 40 minutes incubation in the presence of the inhibitors, most cells displayed cytoplasmic fluorescence clusters and some diffuse cytoplasmic/nuclear staining (panel 5). Once the inhibitors were washed away and serum was added, the majority of the cells became motile again and the La9-gfp fusion protein re-entered the nucleus (panel 6). From these observations we concluded that the C-terminal peptide of *T. brucei* La (RGHKRSRE) contained the NLS, and that La9-gfp was imported into the nucleus via an energy-dependent mechanism. Lastly, these experiments showed that gfp can be used as a suitable reporter for assessing nuclear import of proteins in *T. brucei* cells.

### Nuclear targeting of H2B is mediated by a bipartite NLS

Next, we examined the targeting mechanism of histone H2B of *T. cruzi*, a highly conserved protein that has been shown in other systems to carry an NLS at the amino terminus (Moreland et al., 1987). We fused the region coding for amino acids 2-110 of H2B to the amino terminus of gfp to generate pH2B-gfp (Fig. 3A), and established a stable cell population expressing the corresponding fusion protein. H2B-gfp fluorescence was nuclear, clearly excluded from the nucleolus (Fig. 3A), and co-localized with chromosomal DNA in dividing nuclei (Fig. 3B). This established that *T. cruzi* H2B was capable of directing accumulation of gfp into the nucleus and that the fusion protein was likely to be incorporated into chromatin.

In the yeast *S. cerevisiae*, histone H2B appears to be imported into the nucleus as a heterodimer with histone H2A via the H2A interaction domain located between positions 40-117 at the carboxy terminus (Moreland et al., 1987). A corresponding domain with a high degree of primary sequence conservation is present in *Leishmania* and *T. cruzi* H2B and might perform a similar function in the trypanosomal H2B proteins (Garcia-Salcedo et al., 1994). To analyze whether this



**Fig. 3.** (A) Intracellular distribution of histone H2B-gfp fusion proteins. Stable cell populations expressing H2B-gfp or H2B40-gfp were established and the fluorescence of the corresponding fusion proteins is shown in panels H2Bwt and H2B40, respectively. The fluorescence shown for mutant H2B27-gfp cells is after transient expression with the corresponding plasmid DNA (H2B27). (a and i) Phase images; (d) DIC images; (b,e,j) gfp fluorescence; (c and f) DAPI staining. (g) Overlay of d and e. (h) Overlay of d and f. DIC, differential interference contrast. (B) Co-localization of histone H2B-gfp with chromosomal DNA. Images of dividing nuclei were collected from a stable cell population expressing the histone H2B/gfp fusion protein. Images are from early spindle (1) to after chromosomal segregation (4).

putative H2A interaction domain mediated the import of H2B-gfp, amino acids 41-110 of H2B were deleted, leaving 40 amino acids fused to gfp (Fig. 4, H2B40). H2B40-gfp was nuclear but, unexpectedly, the fluorescence became primarily concentrated in the nucleolus (Fig. 3A, H2B40). Further deletion to amino acid 27 (H2B27, Fig. 4) resulted in cells with

**Fig. 4.** Summary of nuclear accumulation of histone H2B-gfp and of H2B-gfp- $\beta$ -galactosidase fusion proteins. The sequence of amino acids 1-40 of *T. cruzi* histone H2B is given in single-letter code at the top. Diamonds indicate Lys-17, Lys-21, Lys-23, Lys-35 and Arg-32 that are conserved residues between the *T. cruzi* and *Leishmania* histone H2B sequence. Mutant names are indicated on the left. For the deletion mutants the number following H2B delineates the extent of amino-terminal residues. By site-directed mutagenesis lysine residues were changed to asparagine residues (N) and the Arg-32 residue was changed to serine (S). The changes in the various mutants are indicated and unchanged residues are indicated by dots. Nuclear accumulation of the various histone H2B-gfp and H2B-gfp- $\beta$ -galactosidase fusion proteins is indicated on the right. (+), indicates nuclear accumulation in all cells examined; (-), indicates lack of nuclear accumulation; (+/-) indicates partial nuclear accumulation; nd, not determined.

		Nuclear accumulation	
		gfp	$\beta$ -gal
H2B40	MATPKSSSANRKKGGK $\blacklozenge$ SHR $\blacklozenge$ K $\blacklozenge$ PKRTWNVYINR $\blacklozenge$ SL $\blacklozenge$ K $\blacklozenge$ SINNH	+	+
H2B27	.....	+/-	nd
H2B15	.....	-	-
H2B/1	.....N...N...N.....	+/-	-
H2B/3	.....N...N...N.....S...N.....	+/-	nd
H2B/4	.....S.....	+	+
H2B/6	.....N.....	+/-	+/-
H2B/5	.....S...N.....	+/-	+/-

an intermediate phenotype, namely incomplete nuclear accumulation of gfp fluorescence with staining of the cytoplasm, the nucleoplasm and the nucleolus (Fig. 3A, H2B27). Lastly, nuclear accumulation of gfp was abolished when only amino acids 2-15 of H2B were present in the fusion protein (Fig. 4, H2B15). These initial observations established that the amino terminal 40 amino acids of *T. cruzi* H2B were sufficient to mediate nuclear accumulation of gfp and that sequences located between amino acid positions 16 and 40 were required for this phenomenon.

As discussed above for the La protein, nuclear/nucleolar accumulation of H2B40-gfp could be accounted for by diffusion of the fusion protein through the NPC followed by nuclear retention via binding to nuclear/nucleolar components. To verify whether nuclear uptake of H2B40-gfp was NLS-mediated, we increased the molecular mass of the fusion protein beyond the size-exclusion limit of NPCs. This was accomplished by fusing H2B40-gfp to the amino terminus of  $\beta$ -galactosidase ( $\beta$ -gal), a classical reporter for nuclear transport studies, to generate a H2B40-gfp- $\beta$ -gal fusion. Transient expression of this construct resulted in the appearance of the hybrid protein in the nucleoplasm, but not in the nucleolus, whereas gfp- $\beta$ -gal was cytoplasmic (Fig. 5). These observations established that the first 40 amino acids of histone H2B encoded a NLS, and that the nucleolar localization of H2B40-gfp was likely the result of a serendipitous interaction between this fusion protein and a nucleolar component.

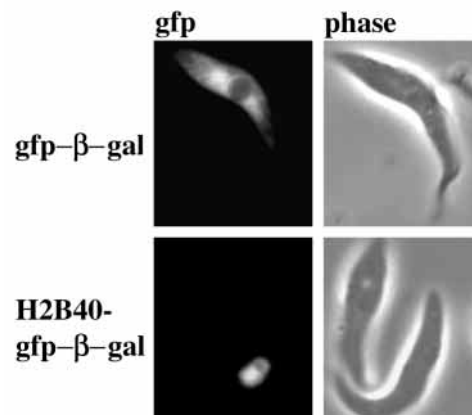
To identify more precisely the residues in histone H2B involved in nuclear targeting, we constructed a series of point mutants shown in Fig. 4 and assayed them by fusion to gfp or to gfp- $\beta$ -gal. Based on the sequence conservation between *T. cruzi* and *Leishmania* histone H2B (Garcia-Salcedo et al., 1994), we selected five basic amino acids for mutagenesis, namely Lys-17, Lys-21, Lys-23, Arg-32 and Lys-35. This analysis showed that a triple mutation of lysines at positions 17, 21 and 23 (H2B/1) led to a defect in nuclear localization and gave rise to cytoplasmic fluorescence in the context of gfp- $\beta$ -gal, and to incomplete nuclear accumulation when fused to gfp. Lys-35 was also part of the NLS, as shown by the incomplete nuclear accumulation of mutants H2B/5 and H2B/6, in the context of either reporter system. In contrast, Arg-32 did not seem to play a role in nuclear import. Taken

together, our results with deletion constructs and site-directed mutagenesis suggest that the trypanosome H2B protein is targeted to the nucleus by a bipartite NLS consisting of two elements enriched in lysine residues.

### Visualization of mitotic cells

The targeting of gfp to nuclear DNA as observed with H2B-gfp, or to the nucleolus in the case of H2B40-gfp, or to the nucleoplasm in the case of La-gfp, allowed visualization of trypanosomes while their nuclei were dividing. Fig. 6A presents a gallery of images of mitotic H2B40-gfp expressing cells arranged in an progressive temporal order from early spindle (panel 1) to after karyokinesis (panel 6) on the basis of DAPI staining and the position of the kinetoplast. In these images gfp fluorescence was coincident with areas of weaker DAPI staining, thus localizing the fusion protein to the nucleolar area. By this analysis nuclear DNA segregation preceded nucleolar separation (panels 3-5). Interestingly, we found that amongst the cells that had completed nuclear division by DAPI staining, about 25% displayed a fluorescent thread connecting the two nuclei (panels 4 and 5, data not shown).

Since the nucleolar localization of H2B40-gfp was



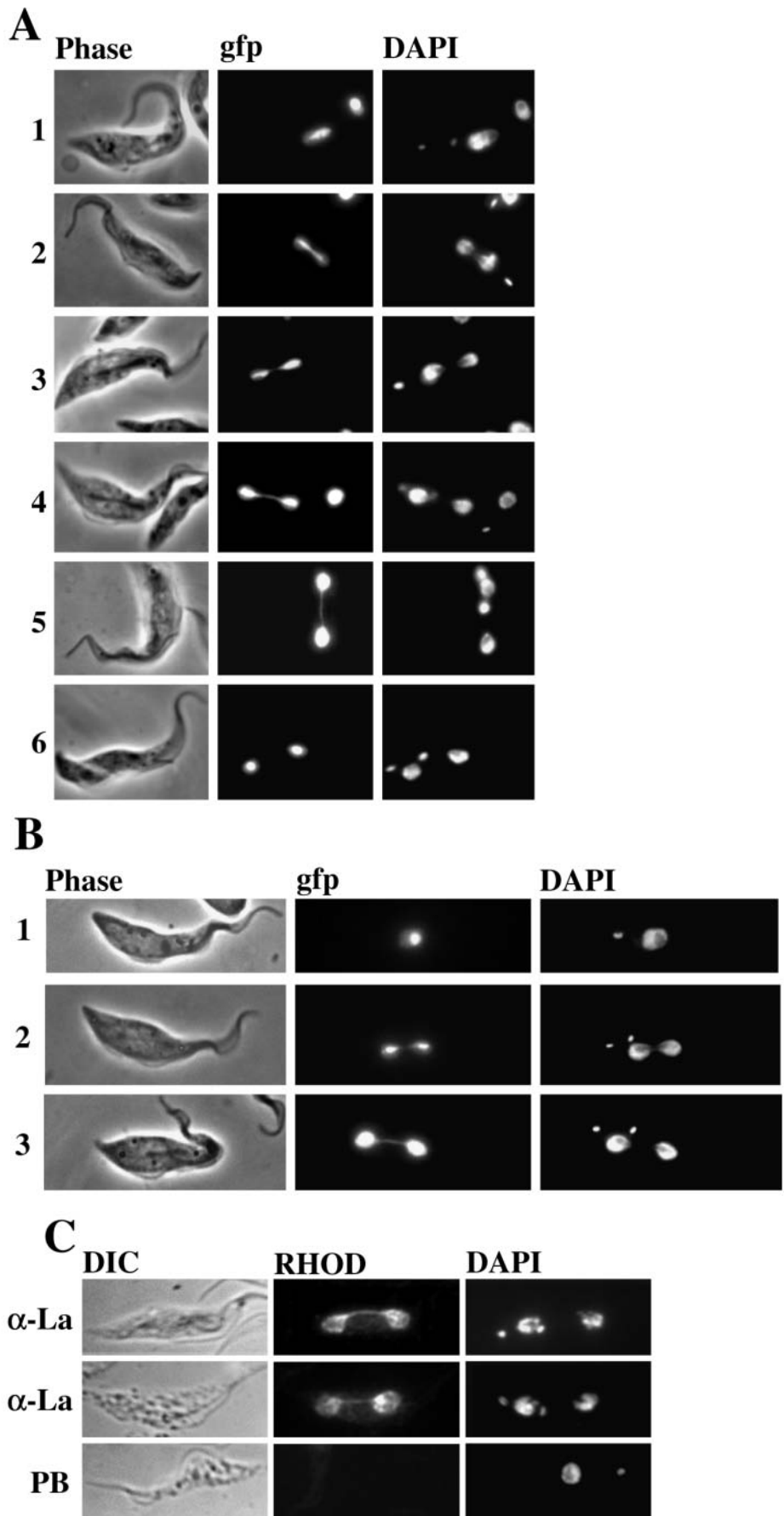
**Fig. 5.** Intracellular distribution of gfp- $\beta$ -galactosidase fusion proteins. Cells were transiently transfected with pGFP- $\beta$ -gal or pH2B40-GFP- $\beta$ -gal, living cells were mounted on microscope slides and gfp fluorescence was recorded.

unexpected we were concerned about the possibility of artifacts, and in particular that the fluorescent thread could be the result of H2B mislocalization. As a first control, we expressed a true nucleolar marker, the *T. brucei* protein fibrillarin (S. Wormsley and S. Baserga, unpublished data), as a fusion protein in the pXSGFPM3FUS vector. Fig. 6B shows representative images of fibrillarin-gfp expressing cells. In this instance, the fluorescence was mainly localized to the nucleolus (panel 1), but there was also some staining of the nucleoplasm. Nucleolar division in these cells was indistinguishable from the one described above for H2B40-gfp-expressing cells. In particular, the same thread-like structure was observed (panel 3). As a second control, we performed indirect immunofluorescence with a rabbit polyclonal serum against the *T. brucei* La protein (Fig. 6C). The La antigen was nucleoplasmic with a lace-like distribution, and a proportion of the mitotic nuclei displayed a thread-like structure similar to that observed by direct visualization of H2B40-gfp in live or fixed cells.

## DISCUSSION

In this study we have documented the existence of both monopartite- and bipartite-type NLS in trypanosomal proteins and that entry of proteins into the trypanosome nucleus is an energy-dependent process. Thus, the signals and machinery for nuclear import in trypanosomes are very much like those in higher eukaryotic organisms. Given that trypanosomes are considered amongst early divergent eukaryotes, this suggests

**Fig. 6.** A proportion of mitotic nuclei are connected by a thread-like structure after chromosome segregation. (A) Gallery of images of cells expressing H2B40-gfp. The gfp staining in 5 is overdone in order to show the thread connecting the nucleoli. (B) A gallery of images of cells expressing fibrillarin-gfp. (C) Indirect immunofluorescence of *T. brucei* procyclic cells with La antiserum. Cells were processed for immunofluorescence as described in Materials and Methods. After reaction with the La antiserum ( $\alpha$ -La panels) or with pre-immune serum (PB panels), cells were decorated with a rhodamine-conjugated goat anti-rabbit secondary antibody (RHOD panels) and stained with DAPI (DAPI panels).



that the components of the import machinery were an early acquisition of the eukaryotic cell.

The signals mediating nuclear import and retention of the La protein have been studied for human and yeast La (Rosenblum et al., 1998; Simons et al., 1996). Both studies employed deletion analysis to pinpoint the NLS. In the case of human La the NLS-containing sequence was localized to the carboxy terminal 28 amino acids, whereas the yeast La seems to contain two regions that can independently target the protein to the nucleus. These regions have been grossly mapped to amino acids 112-224 and 188-275. The latter fragment corresponds to the carboxy terminus of yeast La. In the case of the trypanosome La homologue, the NLS is coincident with the last seven amino acids, similar to what has been reported for the human La. However, the carboxy terminal sequences of the trypanosome and human La bear little similarity except for the presence of charged amino acid residues. The significance of the findings for the yeast La protein is at present unclear and further mutagenesis is needed.

The NLS of the trypanosome histone H2B was localized to the amino terminal forty amino acids. As a comparison, the yeast histone H2B NLS is monopartite and spans amino acids 28-33 of the protein (Moreland et al., 1987). Although we did not systematically mutagenize all the amino acids of the trypanosome histone H2B NLS, the combined results of our deletion and site directed mutagenesis analysis support a bipartite structure. In particular, the localization of mutant H2B/1 demonstrated that Lys-17, Lys-21 and Lys-23 are part of the NLS, but they were not sufficient to direct efficient nuclear localization of the reporter protein, since the mutant H2B27-gfp fusion, containing the first 27 amino acids of H2B, was localized both to the nucleus and the cytoplasm (Figs 3A and 4). Thus, amino acids downstream of position 27 were also implicated as part of the NLS. By site-directed mutagenesis we identified one important residue in this region, namely Lys-35, as shown by the impaired nuclear localization of mutant H2B/6. There are eleven amino acids between Lys-23 and Lys-35 and this spacing is similar to that of the nucleoplasmin NLS, where ten amino acids separate the two blocks of the bipartite NLS (Robbins et al., 1991). In conclusion, it appears that both monopartite and bipartite NLS exist in trypanosome nuclear proteins.

To demonstrate the energy dependency of nuclear import, we incubated living trypanosomes expressing La9-gfp with poisons of ATP synthesis. We observed a peculiar phenomenon, namely that upon exit from the nucleus La9-gfp accumulated in clusters or speckles of fluorescence that localized preferentially to the cell periphery. Once ATP synthesis was allowed to resume, the fluorescence returned to the nucleus and the cells reacquired full motility, demonstrating that this phenomenon was not associated with cell death. At present, we do not understand the molecular basis for the cytoplasmic clustering of La9-gfp and we can only speculate about its functional significance. One possibility is that in trypanosomes nuclear proteins travel on cytoskeletal tracks towards and from the nucleus and that the speckles highlight areas of the cytoskeleton where the tracks begin. Interestingly, in tobacco protoplasts most of the cytoplasmic importin  $\alpha$  coaligned with microtubules and microfilaments (Smith and Raikhel, 1998). In contrast, attempts to block NLS protein import in vitro with cytoskeleton depolymerizing

agents have not been successful (Schmalz et al., 1996). Thus, further experiments are needed to elucidate the identity of the speckles observed in our experiments. Nevertheless, our data demonstrate that as in other eukaryotes nuclear import in trypanosomes is energy dependent.

By fusing gfp to La, histone H2B and mutant derivatives of it, and fibrillarin we were able to tag different components of the nucleus, namely the nucleoplasm, chromosomal DNA and the nucleolus. The nucleolar localization of H2B40-gfp was unexpected and we think this phenomenon is not meaningful since it appeared to be reporter gene dependent.

Observation of trypanosomes expressing the various fusion proteins allowed visualization of mitotic cells. By these analyses we found that a proportion of mitotic nuclei, in which the chromosomal DNA had already segregated, were still connected by a thin thread highlighted by the gfp fluorescence. This thread contained nucleoplasmic and most likely nucleolar components as well, since it was observed with anti-La antibodies, with La-gfp fusion proteins (E. Ullu, unpublished observations) and with fibrillarin-gfp. As determined by DAPI staining, nuclear DNA was excluded from the thread. This was also the case in cells expressing H2B-gfp, where we never observed a thread of gfp fluorescence connecting the two mitotic nuclei. We do not think that this thread is an artifact because it can be observed by immunostaining with anti-La antibodies. Furthermore, a thread connecting mitotic nuclei could also be seen in immunofluorescence images with anti-RNP1 antibody (Manger and Boothroyd, 1998). We propose that cells harboring two nuclei connected by the thread represent a late stage in mitosis that can be placed after chromosomal segregation. The thread connecting mitotic nuclei might correspond to the long (3-4  $\mu$ m) nuclear isthmus observed in electron micrographs of dividing trypanosomes and which contains an array of parallel microtubules (Vickerman and Preston, 1970). The movement of these microtubules might provide the force for pushing the two dividing nuclei away from each other till they reach their specific territory within the cell. If this were the case, the thread might guide the nuclei to their final destination.

This work was supported by grants AI28798 and RO1-GM48410 from the National Institutes of Health to E.U and S.L.W, respectively. E.U is the recipient of a Burroughs Wellcome Scholar Award in Molecular Parasitology. We are grateful to Jay Bangs for providing expression vector pXS2. We thank Jennifer Morehead for contributing to the initial experiments and Anna Polotsky for excellent technical assistance.

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