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Flagellum ontogeny in trypanosomes studied via an inherited and regulated RNA interference system

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SUMMARY

The African trypanosome, *Trypanosoma brucei* possesses a large and unique intraflagellar structure called the paraflagellar rod (PFR). The PFR is composed of 2 major proteins, PFRA and PFRC. We have generated an inducible mutant trypanosome cell line (*snl*-2) that expresses linked inverted copies of a *PFRA* gene, capable of forming a *PFRA* double-stranded (ds) RNA. When expression of this dsRNA was induced, new *PFRA* RNA and PFRA protein quickly disappeared and PFR construction was affected, resulting in cell paralysis. This inducible RNA interference (RNAi) effect was fast-acting, heritable and reversible. It allowed us to demonstrate that PFR proteins are able to enter both mature and growing flagella but appear to concentrate differentially in new flagella because of the construction process. The PFR is constructed by a polar assembly process at the distal end of the flagellum resulting in a stable cytoskeletal structure with low turn-over. The inducible RNAi approach will have widespread applicability in studies of gene function and cellular processes in parasites.

Key words: Cytoskeleton, Flagellum, Paraflagellar rod, RNA interference, Trypanosome

INTRODUCTION

Trypanosomes are motile protozoans responsible for a variety of diseases in a wide range of organisms. One representative of this group, *Trypanosoma brucei* causes African sleeping sickness and these parasites develop in the bloodstream of humans and other mammals as extracellular organisms. Trypanosomes diverged very early in eukaryotic evolution, and exhibit many unique features, both at the cellular (Clayton et al., 1995; Gull, 1999) and molecular level (Vanhamme and Pays, 1995; Lee and Van der Ploeg, 1997). In particular, expression of protein-coding genes is markedly different from other eukaryotes. Genes are transcribed in a polycistronic unit that is then processed by trans-splicing and poly-adenylation. The few promoters characterised so far all seem to use RNA polymerase I and show no significant similarities with other eukaryote promoters (Lee and Van der Ploeg, 1997). RNA polymerase II promoters have not been identified and intergenic sequences do not have promoter activity (Haüsler and Clayton, 1996). In addition, control of gene expression mostly takes place at the post-transcriptional level where the 3′ untranslated region (UTR) plays a critical role for RNA stability and translation.

Considerable progress in understanding parasite biology has been accomplished recently thanks to the development of transfection systems (Clayton, 1999). In *T. brucei*, DNA with linearised ends corresponding to trypanosome sequence is specifically inserted in the genome at this site by homologous recombination (ten Asbroek et al., 1990; Lee and Van der Ploeg, 1990; Eid and Sollner-Webb, 1991). Such homologous recombination facilitates double gene knock-out approaches in these diploid organisms, however, in many studies of phenotype and gene function, a flexible antisense strategy would be a useful alternative method. Unfortunately, expression of antisense RNA from transfected DNA does not have significant effect in trypanosomes (Tovar and Fairlamb, 1996; Clayton, 1999). Two noticeable exceptions have been published recently. First, expression of RNA antisense of the *Leishmania A2* gene (a stage-regulated gene) from an episome resulted in a considerable decrease in the amount of both its mRNA and its protein, reducing the infectivity of the parasite (Zhang and Matlashewski, 1997). These authors suggest that the high number of repeated sequences within the *A2* gene might explain their success. Second, our transformation of *T. brucei* with the aim of expressing antisense RNA of the *PFRA* gene yielded a single clonal mutant called *snl*-1 (Bastin et al., 1998, 1999a). This mutant grew normally but exhibited ablation of the *PFRA* mRNA and PFRA protein, leading to severe alteration of the paraflagellar rod (PFR), a unique lattice-like structure present inside the flagellum of trypanosomes (Bastin et al., 1996a; Maga and Lebowitz, 1999; Bastin and Gull, 1999), and cell paralysis (Bastin et al., 1998, 1999a). The *PFRA* gene (Schlaeppi et al., 1989) encodes one of the 2 major PFR proteins, the other one, PFRC, shares 60% identity with PFRA (Deflorin et al., 1994), but its expression is barely modified in the *snl*-1 mutant (Bastin et al., 1998).

Understanding the molecular mechanisms involved in the production of such phenotypes is essential since it may reveal important features about gene expression in Kinetoplastid parasites.

Our original experiments showed that the effectiveness of the molecular events in the *snl*-1 clone appeared related to the expression of antisense RNA from within the *PFRA* gene cluster. Expression from distant sites in other clones obtained in these experiments produced little or no effect (Bastin et al., 1998, 1999a). Recently, the discovery of a phenomenon called RNA interference (RNAi) has extended conventional views about antisense mechanisms (Fire et al., 1998). Microinjection of a mixture of in vitro synthesised sense and antisense RNA in *Caenorhabditis elegans* worms is much more efficient in blocking the expression of different genes than antisense (or sense) alone. This presence of double-stranded (ds) RNA leads to a specific, potent and rapid degradation of the corresponding mRNA (Montgomery et al., 1998). Mutants incapable of RNAi have recently been identified and indicate the existence of a complex pathway (Cogoni and Macino, 1999a,b; Tabara et al., 1999; Ketting et al., 1999; Grishok et al., 2000). RNAi initiated by dsRNA has been demonstrated in several organisms (reviewed by Fire, 1999) including trypanosomes (Ngô et al., 1998). Consideration of the genotype of the paralysed *snl*-1 mutant suggested that insertion of a strong promoter in the opposite orientation from normal polycistronic transcription of the *PFRA* genes, would provide conditions for in situ expression of sense and antisense RNA. This would permit the capability for local formation of dsRNA, leading to an RNAi effect on the transcripts from downstream genes in that *PFRA* cluster and those from the homologous cluster in this diploid organism. Here we test and verify this hypothesis by experiments which lead to the generation of a new cell line (*snl*-2). This expresses an RNA containing linked copies of sense and antisense *PFRA*, capable of forming a dsRNA, from a tetracycline-inducible promoter. Induction of expression of this *PFRA* dsRNA reproduced PFRA ablation, severe alteration of the PFR assembly and cell paralysis. We show this inducible RNAi system to be fast-acting, heritable and reversible. These properties in the *snl*-2 mutant have allowed us to reveal novel aspects of targeting, construction, turn-over and hierarchy mechanisms operating in forming and mature flagella.

MATERIALS AND METHODS

Trypanosomes, plasmids and transfection

Procyclic *T. brucei* (strain 427) were grown at 27°C in semi-defined medium containing 10% fetal calf serum. Plasmids pHD430 (Wirtz and Clayton, 1995), pHD451 (Biebinger et al., 1997) and pαT6451 (Bastin et al., 1998) have been described. The pαT6451 was linearised through the unique *Nsi*I restriction site for insertion in the procyclin locus, or through *Not*I for insertion in the rDNA spacer locus or through *Bsm*I for insertion within one of the *PFRA* genes. The 3′ UTR of the last *PFRA* gene (529bp, nt 4509 to 5039; Schlaeppi et al., 1989) was PCR amplified from genomic DNA using 5′-GGAAGCTT-GATTGTGTACTGTAATTG-3′ as forward primer (*Hin*dIII site underlined) and 5′-GGGGTACCAACACGGATATATACG-3′ (*Kpn*I site underlined) as reverse primer, cloned in a pBluescript T vector and cut out with *Not*I and *Kpn*I for insertion in the corresponding sites of pαT6451, generating the plasmid p3′PFRAαT6451. This plasmid was linearised through the unique *Bbr*PI restriction site for insertion in the 3′UTR of the last *PFRA* gene of a cluster. The pααPFRA430 was assembled by ligating the nearly full-length copy of the *PFRA* gene (nt 78-1800) cut out from the pPFRATAGSK (Bastin et al., 1996b) with *Hin*dIII and *Bam*HI in the plasmid pPFRATAG430 (Bastin et al., 1999b), an inducible trypanosome expression vector containing the Ty-1 epitope tagged copy of the full length *PFRA* gene, also digested with *Hin*dIII (nt 1 of the *PFRA*) and *Bam*HI (nt 22 of the epitope tag sequence). Therefore, the pααPFRA430 plasmid contains the last 1722 bp from the *PFRA* gene in the antisense orientation, 12 bp from the epitope tag sequence in the antisense orientation, 12 bp from the epitope tag sequence in the sense orientation and the last 1722 bp of the *PFRA* gene in the sense orientation. This plasmid was linearised through the unique *Eco*RV restriction site for insertion in the rDNA spacer or through the unique *Sal*I site for insertion in the actin locus. DNA was purified from cesium chloride gradients or Qiagen resin. Plasmids were linearised by single restriction digest and introduced in trypanosomes by electroporation (Beverley and Clayton, 1993). Resistant cells were selected as described (Bastin et al., 1999b). For inducible expression of *PFRA* dsRNA, the pααPFRA430 plasmid was integrated in the opposite strand of the rDNA spacer (known to be transcriptionally silent) in trypanosomes expressing the tet-repressor (Wirtz and Clayton, 1995; Bastin et al., 1999b). Transformants were subcloned by limiting dilution in the absence of tetracycline. For screening, wells were duplicated and grown either with or without 1 μg of tetracycline per ml for 24 hours. The clone exhibiting best control of expression was subcloned a second time and named *snl*-2. For induction experiments, *snl*-2 were grown at 3×10^6 cells per ml and incubated with 1 μg tetracycline per ml for the indicated periods of time. For reversibility experiments, *snl*-2 trypanosomes induced for 48 hours were washed 3 times and resuspended in fresh medium without tetracycline.

Immunofluorescence and immunoblotting

Monoclonal antibodies L8C4 (recognising PFRA) (Kohl et al., 1999) and TAT-1 (anti-tubulin, Woods et al., 1989) were used as hybridoma supernatants. For immunofluorescence, trypanosomes were spread on poly-L-lysine coated slides, fixed in methanol and processed as described (Sherwin et al., 1987). Slides were examined with a Zeiss Axioskop or a Leica DMRXA microscope. Images were captured using a cooled slow scan CCD camera and processed in Adobe Photoshop. For immunoblotting, 10^7 trypanosomes were washed twice in PBS, resuspended in Laemmli buffer in the presence of proteases inhibitors and boiled for 10 minutes before SDS-PAGE. Proteins (10 μg per lane) were transferred to nitrocellulose membranes and stained with India ink before processing. Final detection was carried out using ECL according to manufacturer's instruction (Amersham).

RESULTS

Insertion of inverted promoters within the *PFRA* genes cluster leads to PFRA ablation

Our original Southern blot analysis of genomic DNA from *snl*-1 trypanosomes showed that the pαT6451 plasmid was not inserted at the expected targeted procyclin locus but was present within one of the *PFRA* alleles (Bastin et al., 1998). This was confirmed by extensive Southern blotting using different probes such as the *PFRA* gene, the hygromycin-resistance gene and the pGem backbone plasmid (not shown). The plasmid had presumably been integrated at this site by a rare recombination event (Fig. 1A). This view was strengthened by analysis of the other drug-resistant transformants from this original experiment since they all

showed the expected integration at the procyclin locus. None of these exhibited the paralysed phenotype or lack of PFR structure. Trypanosomes are diploid and the insertion in *snl*-1 had occurred at only one allele, the other one being unchanged as compared to wild-type genomic DNA (Fig. 1A). Such an insertion results in the introduction of 2 promoters in the opposite orientation relative to the *PFRA* genes, therefore providing the capability for production of 2 *PFRA* antisense RNA molecules (Fig. 1A).

We were able to reproduce the *snl*-1 genotype and its paralysis phenotype by deliberately inserting the pαT6451 plasmid within one of the 2 *PFRA* genomic clusters. This was achieved by linearisation in the *PFRA* sequence at the *Bsm*I unique restriction site. Repeated attempts to accomplish ablation of PFRA expression and a paralysis phenotype by antisense expression from distant sites in the genome such as the procyclin or rDNA gene loci (Fig. 1B) were unsuccessful. Similarly no paralysis phenotype resulted from expression of antisense immediately downstream of the *PFRA* gene cluster (Fig. 1C). All the experiments suggested that the effect was only produced by insertion into the *PFRA* gene cluster, with promoters operating in the opposite direction to endogenous transcription (insertion of a plasmid similar to pαT6451 but with promoters integrated in the same direction as *PFRA* transcription did not produce PFRA ablation, not shown). This insertion could lead to *in situ* concomitant and overlapping production of sense and antisense *PFRA* RNA, likely to form *PFRA* dsRNA and hence produce the RNAi effect that has been demonstrated in several species (reviewed by Fire, 1999).

Inducible expression of *PFRA* dsRNA leads to regulatable cell paralysis

To address our hypothesis of the importance of in situ, overlapping, production of sense and antisense *PFRA* RNA, we constructed the pααPFRA430 plasmid, able to express an RNA containing linked sense and antisense copies of the *PFRA* gene, potentially forming a dsRNA in a hairpin structure (Fig. 1D). To control the expression of this RNA, we used a tetracycline-inducible promoter (Wirtz and Clayton, 1995). In these conditions the modified trypanosome promoter will lead to regulatable transcription of the downstream gene dependent on the presence of tetracycline. The pααPFRA430 plasmid, linearised with *Eco*RV, was transfected into trypanosomes expressing the tet-repressor and inserted in the inverted rDNA spacer. Resistant populations were cloned in the absence of tetracycline and these cells exhibited the classic aspect of wild-type trypanosomes with normal growth and motility. However, when cultures were grown in the presence of tetracycline, they still divided normally but exhibited serious motility defects similar to those observed for the *snl*-1 mutant. This was the case for all the transformants obtained with the pααPFRA430 plasmid (more than 50 clones from 4 different transfection experiments). Consequently, these cells were called *snl*-2. The paralysed phenotype was also observed in transformants where the pααPFRA430 plasmid, linearised with *Sal*I, was inserted downstream of the actin gene. Reduction in motility was also observed in the related parasite *L. mexicana* after double gene knock-out of the *PFRA* homologue (Santrich et al., 1997; Maga et al., 1999). In contrast, transfection with the control plasmid pPFRAPFRA430 (2 tandem sense *PFRA* copies) yielded transformants that all behaved exactly like wild-type trypanosomes in both induced and non-induced conditions.

Immunofluorescence with the anti-PFRA antibody L8C4 (Kohl et al., 1999) performed on *snl*-2 trypanosomes grown without tetracycline showed the normal bright staining of the flagellum in all cells (Fig. 2A), whereas all the cells from induced samples exhibited no signal at all. This indicates the absence of a normal PFR. In contrast, expression levels of other proteins such as tubulin, procyclin or protein components of the basal body, the nucleus and the flagellum attachment zone filament were not modified (data not shown). Immunoblot analysis with L8C4 showed that PFRA was totally absent in *snl*-2 samples induced for 48 hours, in contrast to the non-induced situation (Fig. 2B). Northern blots were carried out on total RNA extracted from *snl*-2 trypanosomes induced for 0, 4 or 72 hours (Fig. 2C). The *PFRA* mRNA was barely visible in samples induced for only 4 hours and was not detectable at all after 3 days of induction, when the paralysis phenotype was extreme. A sense probe failed to detect any *PFRA* antisense molecule and none of the probes could detect an RNA corresponding to the expected total mass of the *PFRA* dsRNA.

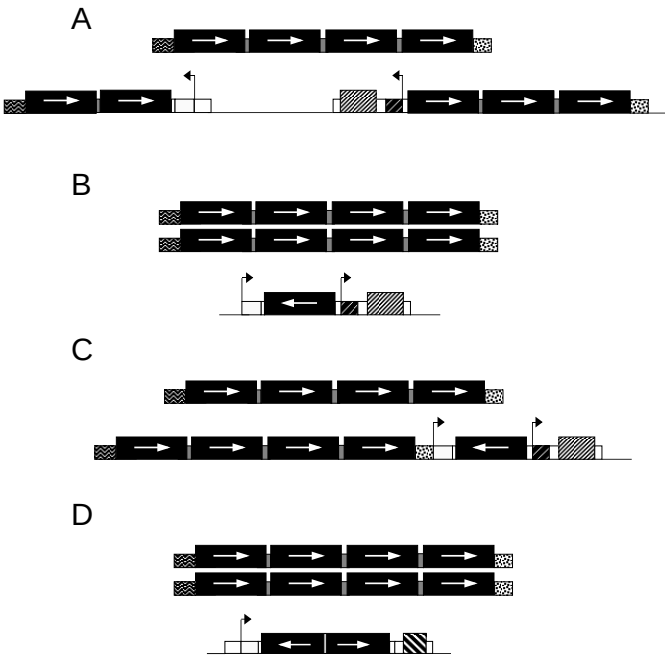

Fig. 1. Genotypes of trypanosome cell lines used in this study. The large black boxes represent *PFRA* gene coding sequences, with the white arrow indicating orientation of the coding sequence. In wild-type trypanosomes, 2 clusters of 4 *PFRA* genes (Deflorin et al., 1994) are present (trypanosomes are diploid). The 5′UTR preceding the first *PFRA* gene, the intergenic sequences and the 3′UTR downstream of the last *PFRA* gene of the cluster are represented as small boxes, as well as processing signals present in the transfected plasmids (Wirtz and Clayton, 1995; Biebinger et al., 1997). The thin line represents the bacterial plasmid sequence. Insertion of the pαT6451 plasmid (A) within the *PFRA* gene cluster (*snl*-1 genotype) or (B) in a different genomic site such as the procyclin or the rDNA locus. (C) Insertion of the p3′PFRAαT6451 immediately downstream of the last *PFRA* gene of one cluster. (D) Insertion of the pααPFRA430 in the rDNA spacer for expression of *PFRA* dsRNA (*snl*-2 genotype).

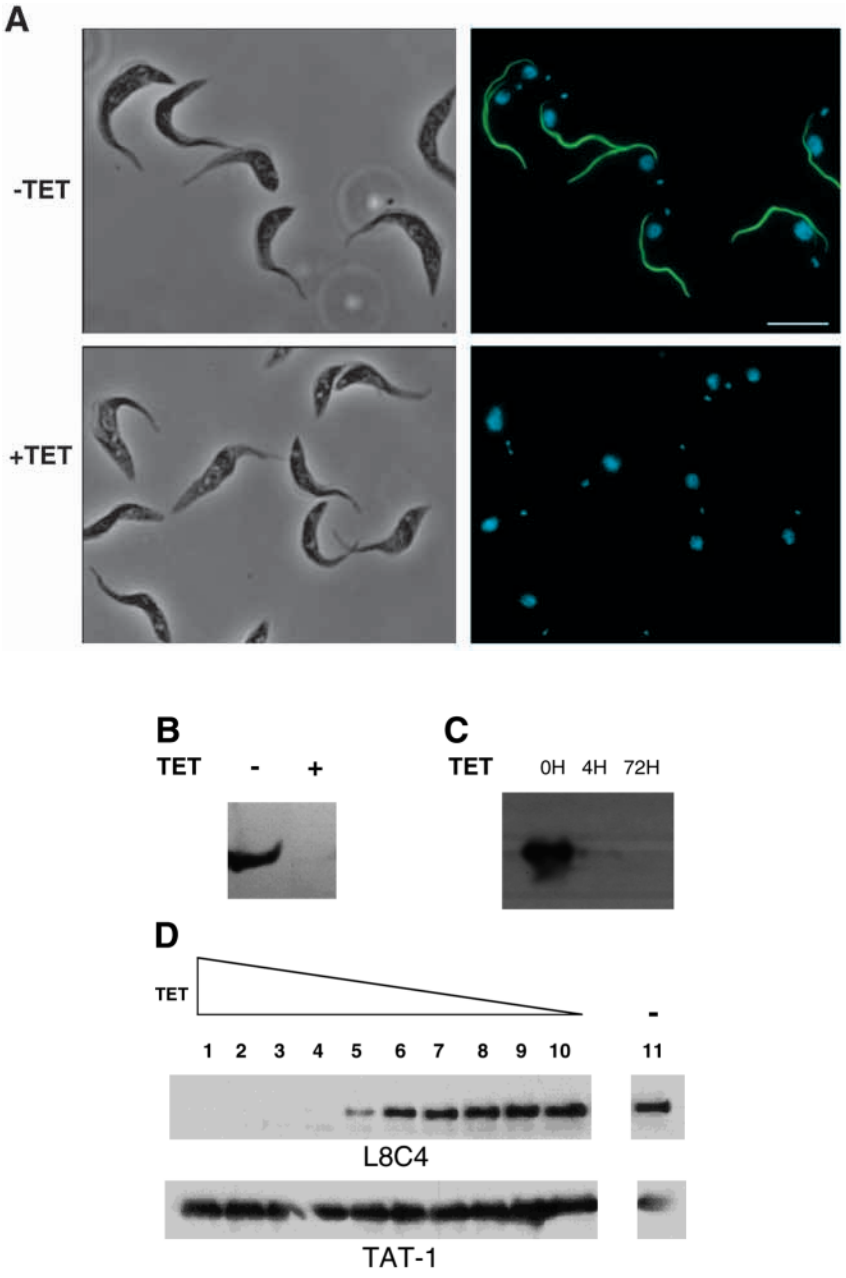

Fig. 2. Expression of *PFRA* dsRNA leads to ablation of *PFRA* RNA and PFRA protein in *snl*-2 trypanosomes. (A) Immunofluorescence of *snl*-2 trypanosomes with the L8C4 antibody, the left image shows the phase contrast picture and the right panel shows the fluorescence signal (green), merged to DAPI staining (blue). Normal signal is displayed by non-induced cells (top) but no signal at all is observed in samples induced for a week (bottom). Bar, 10 μm. (B) Western blots of total protein samples of *snl*-2 trypanosomes grown without or with tetracycline for 48 hours. Proteins were blotted and probed with the anti-PFRA specific antibody L8C4. The normal amount of PFRA is present in non-induced *snl*-2 but no PFRA can be detected in the induced population. (C) Northern blots of total RNA samples from *snl*-2 cells induced for 0, 4 or 72 hours probed for *PFRA* sense RNA, showing rapid disappearance of the *PFRA* RNA. (D) A critical level of expression of the *PFRA* dsRNA is required for ablation of PFRA expression. Western blots of total protein samples (10 μg protein per lane) of *snl*-2 trypanosomes induced for 48 hours in the presence of decreasing concentrations of tetracycline (per ml: 1, 1 μg; 2, 0.1 μg, 3, 0.01 μg, 4, 1 ng; 5, 0.1 ng; 6, 0.01 ng; 7, 1 pg, 8, 0,1 pg; 9, 0.01 pg, 10, 1 fg; 11, no tetracycline). Proteins were blotted and probed with L8C4. The membrane was stripped and reprobed with the anti-tubulin antibody TAT-1 as a loading control.

Stability and titration of the RNAi effect

The paralysis phenotype was exhibited by the *snl*-2 cells for at least 3 months (>300 generations), as long as they were maintained in the presence of tetracycline. In protein expression studies, the tetracycline-repressor system allows titration of the amounts of RNA and consequent protein by modulating the tetracycline concentration in the medium (Wirtz and Clayton, 1995). We grew *snl*-2 cells for 48 hours in the presence of incremental series of tetracycline concentrations (Fig. 2D). When grown with 1ng-1μg of tetracycline per ml, all the *snl*-2 cells were paralysed and the PFRA protein could not be detected on a western blot (lanes 1-4). An abrupt change was observed at 0.1 ng per ml, where most cells appeared to be paralysed, but with a minority of swimming trypanosomes. Only a weak PFRA band was detected on the immunoblot (lane 5) and immunofluorescence of such populations revealed a mixture of brightly stained cells with others that fail to produce any signal at all (not shown). Only a few 'intermediates' or weakly labelled cells were identified. Finally, when *snl*-2 were incubated with 0.01 ng of tetracycline per ml or less, all the cells behaved like wild-type and exhibited the normal amount of PFRA (lanes 6-10), as observed in non-induced samples (lane 11). Therefore, it appears that there is a critical concentration effect operating in this RNAi induction system above which a maximum phenotype is accomplished.

Rapid RNAi induction reveals flagellum assembly processes

In trypanosomes, the flagellum is attached along the length of the cell body. Our previous studies of the trypanosome cell cycle show that the old flagellum remains in place whilst the new one is constructed, always at the posterior end of the cell (Sherwin and Gull, 1989a). PFR construction in the new flagellum starts at around 0.52 of the unit cell cycle (Woodward and Gull, 1990) and closely follows the flagellar axoneme in wild-type trypanosomes. DAPI staining shows that the kinetoplasts (mitochondrial DNA) are segregated first, followed by mitosis in the nucleus. Hence, a trypanosome which has 2 kinetoplasts and 2 nuclei (or 2K2N) will be close to the end of its 8.5 hour cell cycle and will also possess both an old and a new flagellum (Sherwin and Gull, 1989a). So, if one views the 2K2N cells within the population at various points during an induction experiment, one can assess the effect of *PFRA* dsRNA expression on PFRA protein levels, localisation and the consequences for flagellar construction.

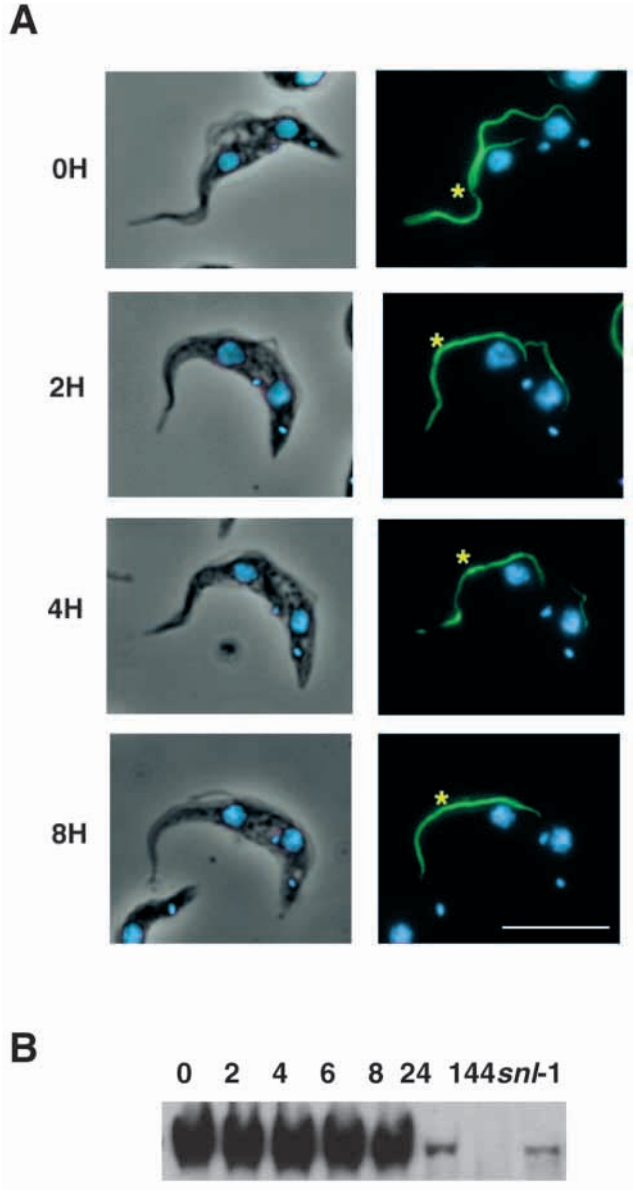

Fig. 3. Time-course induction of expression of *PFRA* dsRNA shows rapid inhibition of new PFRA production in *snl*-2 cells and reveals how PFRA is incorporated in the new flagellum. (A) *snl*-2 were fixed after the indicated period of induction and processed for immunofluorescence with the L8C4 antibody. The left image shows the phase contrast picture merged to DAPI staining (blue), and the right panel shows the fluorescence signal (green), merged to DAPI staining (blue) as a visual aid. 2K2N cells, where the elongation of the new flagellum is almost complete, are shown. The new flagellum is always present at the posterior end of the cell (right-hand side of the picture) and its distal end is indicated by the yellow star. Whereas PFRA is present throughout the full length of the new flagellum in uninduced cells, it is only localised to its proximal part in cells induced for 2 and 4 hours and is totally absent in cells induced for 8 hours (total time for synthesis of new PFR is around 5 hours). Bar, 10 μm. (B) Expression of the *PFRA* dsRNA leads to disappearance of PFRA. Western blots of total protein samples of the *snl*-2 cell line induced to express the *PFRA* dsRNA for 0, 2, 4, 6, 8, 24 hours or 6 days and of *snl*-1 as a comparison. Membranes were probed with the anti-PFRA antibody L8C4. Stripping and probing with the anti-tubulin TAT-1 confirmed equal loading (not shown).

snl-2 cells induced to express the *PFRA* dsRNA were sampled over a 24 hour period and were processed for immunofluorescence with the anti-PFRA antibody (Fig. 3A). In each sample, we concentrated on the 2K2N cells since these provided an indicator, in the same trypanosome, of the effect of PFRA disappearance on new and existing flagella. The RNAi effect on PFRA expression was already distinguishable after only 2 hours of induction. At this time, 2K2N cells showed a lack of PFRA staining at the distal tip of their new flagellum (Fig. 3A). This was more pronounced in such cells after 4 hours of induction (compare with 0 hour in Fig. 3A). 2K2N trypanosomes in populations induced for 8 hours showed an absence of PFRA in their new flagellum. In all cases, PFRA present in the old flagellum was not noticeably affected.

Western blot analysis was performed on such samples and probed with the anti-PFRA antibody L8C4 (Fig. 3B). In non-induced cells, a strong band was detected that only showed a slight reduction after 8 hours of induction (approx. one cell cycle period), since PFRA protein synthesised before the induction was still present in the old flagella of many cells. A considerable difference was observed after further cell divisions and at 24 hours only a weak band remained. After induction for a week, no PFRA band could be detected in *snl*-2 samples, in contrast to the original *snl*-1 mutant where a small amount of PFRA was still present.

Reversibility of RNAi and reacquisition of flagellar structure

To see whether RNAi effects were reversible, *snl*-2 trypanosomes were induced to express *PFRA* dsRNA for 48 hours, then tetracycline was removed from the medium and the paralysed cells were washed extensively (Fig. 4A). Low amounts of PFRA could already be detected 2 days after removal of the inducer, but motility was not restored at that stage. One day later, the amount of PFRA was similar to that observed in the non-induced control and cells displayed normal motility. To make sure that such results were not due to the emergence of revertant cells, this population was grown in the presence of tetracycline and this again led to PFRA ablation and cell paralysis (Fig. 4A). Thus the *snl*-2 trypanosomes represent a conditional mutant, lacking PFRA and being paralysed when RNAi is induced whilst having a wild-type phenotype under non-induced conditions. Such a system allows us to ask what constraints are placed on building portions of a flagellum and what dependency relationship exist? Is construction of a PFR only possible when the flagellar axoneme is being elaborated or can the complex PFR structure be assembled in mature flagella which lack such a component? To address these issues, we washed tetracycline out of an induced, paralysed, *snl*-2 culture and viewed the cells by immunofluorescence microscopy. We examined trypanosomes at all stages of the cell cycle at time intervals over a 72 hour period. As before, cells having an old and a new flagellum were particularly informative. Essentially, we asked whether PFRA returned to both flagella or was only incorporated in the new flagellum. The answer appears to be the former (Fig. 4B). We did not at any time point see cells with one bright flagellum and one unstained flagellum. Rather, regardless of the individual cell to cell variation as the population gained PFRA and motility, both old and new flagellum within each cell

showed essentially the same level of staining (Fig. 4B). This experiment also shows that PFRA protein can access equally both a mature and an elongating flagellum.

DISCUSSION

Ablation of target gene expression dependent on dsRNA formation

Our work strongly suggests that overlapping expression of sense and antisense *PFRA* RNA is required for successful ablation of gene expression in trypanosomes. In contrast, expression of antisense *PFRA* RNA alone did not lead to PFRA ablation, even when produced immediately downstream of the *PFRA* genes. These latter results are similar to reports of expression of large amounts of antisense RNA of the trypanothion reductase gene in *Trypanosoma cruzi* and in *T. brucei* that in each case did not affect significantly protein expression (Tovar and Fairlamb, 1996; Clayton, 1999).

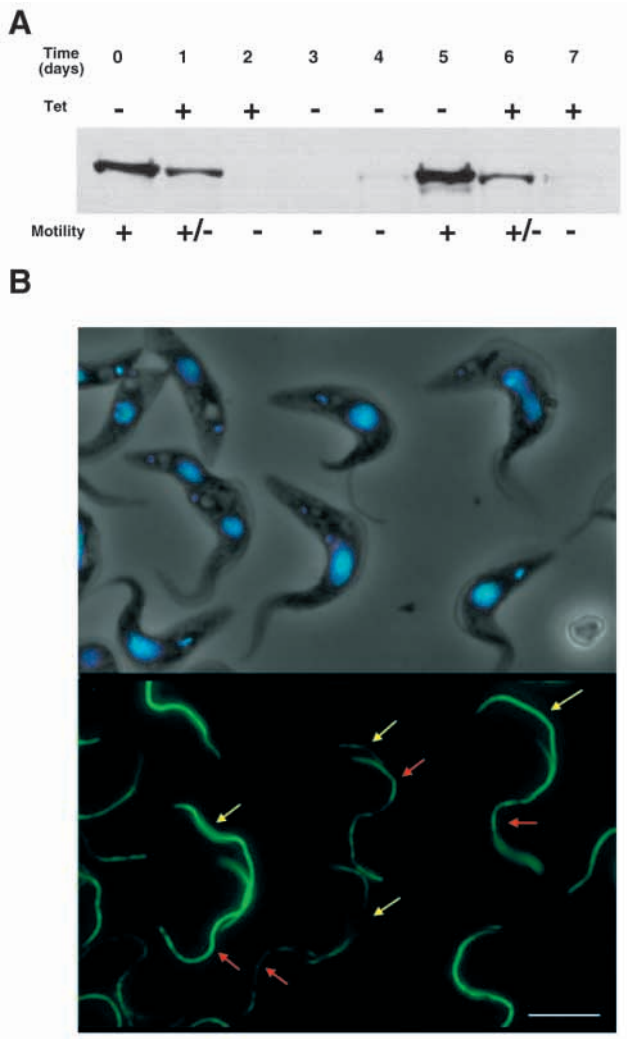

Fig. 4. Reversibility of the RNAi effects on PFRA expression reveals how PFRA is reacquired. (A) *snl*-2 trypanosomes were grown with or without tetracycline as indicated. Samples were analysed by immunoblotting using the L8C4 monoclonal antibody. (B) *snl*-2 trypanosomes were induced to express *PFRA* dsRNA for 48 hours, then washed and allowed to grow in the absence of tetracycline for 64 hours. At that point, *snl*-2 cells were fixed and processed for immunofluorescence with the L8C4 antibody. The top image shows the phase contrast picture merged to DAPI staining (blue), and the bottom panel shows the fluorescence signal (green), PFRA protein is reacquired equally throughout both the new (yellow arrow) and the old flagellum (red arrow) of each individual cell, whatever its total amount of PFRA. Bar, 10 μm.

In contrast, in the *snl*-1 mutant, the insertion of the pαT6451 plasmid results in the integrated promoter potentially driving the transcription of two antisense *PFRA* RNA molecules lacking either a valid 3′ UTR, or both 5′ and 3′ UTR (Fig. 1A). In addition, we assume that the endogenous, distant promoter 5′ to this *PFRA* gene cluster, responsible for normal transcription, is still active. This will result in polycistronic transcription of sense RNA from a total of 5 *PFRA* genes, however, the second gene would lack a valid 3′UTR and the third gene a 5′UTR (Fig. 1A). In such conditions, these RNAs might not be processed immediately and could anneal with each other or with normal *PFRA* sense RNA and form *PFRA* dsRNA. The presence of dsRNA has been shown to lead to potent and specific degradation of the corresponding mRNA in several organisms (Fire, 1999) including trypanosomes (Ngô et al., 1998).

In the *snl*-2 cell line, expression of an RNA molecule containing both sense and antisense of the *PFRA* gene produces even more efficient PFRA ablation than the original *snl*-1. Such an RNA is highly likely to form a stable dsRNA, that could initiate RNAi even when expressed from genomic sites distant from the endogenous *PFRA* genes. It now remains to be seen whether the methodology could be applied to other genes in trypanosomes. In the related parasite *L. donovani*, expression of antisense RNA of the *A2* gene produced successful A2 protein expression ablation (Zhang and Matlashewski, 1997). However, in these experiments, the construct used to express the *A2* antisense RNA was flanked by 2 copies of the *A2* 3′UTR in inverted orientation. With hindsight it is likely that these are able to form *A2* dsRNA and to initiate RNAi.

Inducible RNAi in trypanosomes

Our development of an inducible RNAi system that is particularly effective at massively reducing target protein expression reveals interesting aspects of the RNAi phenomenon. First, the system is very stable giving long term control whilst inducer is present (>300 generations). Second, our studies show that is very fast acting, and a reversible phenomenon. This latter result suggests that RNAi consequences are not irreversible modifications at the DNA levels as initially suggested (Wagner and Sun, 1998). In addition, a critical level of *PFRA* dsRNA appears to be needed to produce the full molecular and cellular phenotype of the *snl*-2 mutant. This threshold effect could be significant when taken together with evidence pointing towards amplification/enzymatic steps in RNAi operation (Fire et al., 1998). This amplification step is also underlined by the recent discovery of the central role of a putative RNA-dependent RNA polymerase in mutants disabled in RNAi (Cogoni and Macino, 1999a; Smardon et al., 2000).

Flagellum ontogeny

The inducible RNAi system for PFRA acts as an effective conditional PFRA mutant allowing the interrogation of flagellar ontogeny. Under normal circumstances, the PFR is constructed at the same time as the axoneme in the new flagellum. Our studies of how the *snl*-2 mutant population reacquires PFRA show that PFR proteins can access both an existing as well as a growing flagellum. This extends and clarifies our earlier observation (Bastin et al., 1999b) that an epitope tagged PFRA protein was preferentially located in the new flagellum. Given these new results, it appears that flagellar proteins can access equally both old and new flagella but are found preferentially in the new because of the assembly process. Furthermore reacquisition of a full PFR by old flagella in *snl*-2 suggests that such flagella still maintain an intraflagellar transport and assembly system (Cole et al., 1998; Pazour et al., 1998, 1999; Porter et al., 1999; Rosenbaum et al., 1999).

Moreover, the full PFR is reacquired by old and new flagella equally, showing that full flagellum construction is not necessarily required to be concomitant with original axoneme construction. Whilst we have studied the relationships between two diverse flagellum structures, these results are reminiscent of how different components of the axoneme are reacquired. In *Chlamydomonas*, various authors have shown that specific components can be added to existing flagella, so rescuing the mutant phenotypes (Lefebvre and Rosenbaum, 1986).

The fast-acting RNAi action on PFRA expression whilst the cell is constructing a new flagellum reveals interesting aspects of flagellum assembly in trypanosomes. First, the pattern of inhibition of PFR assembly in the new flagellum shows that assembly is polar and occurs at the distal end. This is similar to the pattern of axoneme assembly (Witman, 1975; Sherwin and Gull, 1989b; Johnson and Rosenbaum, 1992). The PFR in the old flagellum is essentially unchanged by PFRA ablation suggesting a high level of stability and low turn-over. This result confirms in a novel manner that found by ourselves when we studied patterns of insertion of epitope tagged PFRA proteins into new and old flagella (Bastin et al., 1999b).

Finally, this effective, fast-acting, heritable and reversible RNAi system has proven to be extremely valuable for dissecting aspects of flagellum ontogeny in trypanosomes. Given the diploid nature of this and other parasites, this approach is likely to be of major use in studies as diverse as revealing essential cell functions and target verification in drug development.

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