The Major Component of the Paraflagellar Rod of *Trypanosoma brucei* Is a Helical Protein That IsEncoded by Two Identical, Tandemly Linked Genes

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Abstract. The flagellum of the parasitic hemoflagellate *Trypanosoma brucei* contains two major structures: (a) the microtubule axoneme, and (b) a highly ordered, filamentous array, the paraflagellar rod (PFR). This is a complex, three-dimensional structure, of yet unknown function, that extends along most of the axoneme and is closely linked to it. Its major structural component is a single protein of 600 amino acids. This PFR protein can assume two different conformations, resulting in two distinct bands of apparent molecular masses of 73 and 69 kD in SDS–gel electrophoresis. Secondary structure predictions indicate a very high helix content. Despite its biochemical similarity to the intermediate filament proteins (solubility properties, amino acid composition, and high degree of helicity), the PFR protein does not belong in this class of cytoskeletal proteins. The PFR protein is coded for by two tandemly linked genes of identical nucleotide sequence. Both genes are transcribed into stable mRNAs of very similar length that carry the mini-exon sequence at their 5′ termini.

*Trypanosoma brucei*, the causative agent of human sleeping sickness and a complex of veterinary diseases in large parts of Africa, is a uniflagellate protozoon (of the Kinetoplastida order and Trypanosomatida family). Its single flagellum, which arises from within the cell body, emerges through a “flagellar pocket” and extends along the outer cell surface toward and beyond the anterior end of the cell. The flagellum contains a microtubular axoneme of the canonical nine plus two configuration. Besides this axoneme, the trypanosomal flagellum contains a second prominent structure, the paraflagellar rod (PFR) complex (Fuge, 1969; Vickerman and Preston, 1976).

PFR structures have been described for three large groups of flagellates: the kinetoplastids, the euglenoids, and the dinoflagellates (Cachon et al., 1988). They are always highly ordered lattices of fibrous proteins that are located inside the flagellum and assume a fixed orientation with respect to the microtubular axoneme (Souto-Padron et al., 1984). However, preliminary structural studies have already indicated substantial differences in the architecture of the PFR complexes from kinetoplastids and euglenoids (DeSouza and Souto-Padron, 1980; Hyams, 1982).

Despite its structural prominence, nothing is known about the function of PFR (Cachon et al., 1988). Piccinni et al. (1975) observed an ATPase activity in the PFR of *Euglena gracilis* and proposed a function for this structure in flagellar motility. For trypanosomatids, such a function appears less probable. Two species of trypanosomatids, *Crithidia deanei* and *C. oncopelti*, have been observed that lack the PFR structure entirely (Freymüller and Camargo, 1981). However, a comparison of the wave patterns of beating flagella from these organisms with those from a *Crithidia* species that does contain a PFR complex revealed no differences, suggesting that PFR is unlikely to be involved in flagellar mechanics (Goldstein et al., 1970; Johnson et al., 1979).

The overall structure of the PFR complex was found to be very similar in many trypanosomatids such as *T. congolense* (Evans et al., 1979), *Herpetomonas mariadeanei* (Freymüller and Camargo, 1981), *C. fasciculata* (Russell et al., 1983), *Phytomonas davidi*, *H. megaseliae* (Farina et al., 1986), *T. cruzi*, *H. samueli pessoi*, *Leptomonas samueli*, *C. harmosa* (DeSouza and Souto-Padron, 1980), and *T. brucei* (Schneider et al., 1987).

Biochemical and immunological analyses of the PFR structure of several trypanosomatids by different authors have suggested that its major components are two immunologically closely related proteins of apparent molecular masses of ~73 and 69 kD. Two proteins of similar molecular masses have also been detected in *E. gracilis*, another organism containing a PFR structure (Hyams, 1982). These two proteins are immunologically related to the trypanosomal PFR proteins (Gallo and Schrevel, 1985).

The present report describes the isolation and characterization of the genetic locus coding for the major PFR protein. The results demonstrate that, in contrast to the general belief, the major structural component of the PFR is a single protein (PFR protein) of 600 amino acids, which corre-
sponds to the lower molecular mass (69 kD) PFR component described in the literature. The second, more slowly migrating (73-kD) protein band is a reduction-induced derivative thereof. The PFR protein is coded for by two closely linked, tandemly arranged genes of identical nucleotide sequence.

**Materials and Methods**

**Growth of Cells**

Trypanosoma brucei, stock STIB 366 were grown in SDM-79 medium (Brun and Schönberger, 1979) at 26°C. The other Trypanosoma strains—T. brucei rhodesiense, T. brucei gambiense, T. congolense, T. simiae, T. cruzi, and T. rangeli—as well as Leishmania major, L. donovani, and C. fasciculata were gifts from the Tropeninstitut, (Basel, Switzerland). C. deanei (ATCC 30255) and C. oncophel (ATCC 12982) were purchased from the American Type Culture Collection (Rockville, MD). They were grown at 25°C in media according to the manufacturer. E. gracilis was a gift from M. Schärer and Chlamydomonas reinhardtii was a gift from A. Boscetti (Departments of Plant Physiology and Biochemistry, respectively, University of Bern, Bern, Switzerland). Tetrahymena pyriformis was provided by R. Peck (Department of Protozoology, University of Geneva, Geneva, Switzerland). Dictyostelium discoideum was from R. Parish and Giardia lamblia was from B. Gottstein (Departments of Plant Biology and Parasitology, respectively, University of Zurich, Zurich, Switzerland).

**Isolation of Proteins**

For total proteins, 10 ml of cells were harvested during exponential growth: i.e., at densities of ~5 × 10^6 to 1 × 10^7 per ml. The cells were washed once in 10 mM morpholino propane sulfonic acid, pH 6.9, 1 mM MgTA, 1 mM MgSO_4, 250 mM sucrose and then solubilized in 500 μl sample buffer (Laemmli, 1970). After boiling for 10 min, 20–30 μl were analyzed on polyacrylamide gels.

Cell fractionations for isolating cytoskeletal and flagellar proteins were performed as described (Schneider et al., 1987, 1988). The resulting protein fraction consists predominantly of the axonemal α- and β-tubulin and of two proteins that migrate in SDS-gel electrophoresis with apparent molecular masses of 73 and 69 kD (Gallo and Schrevel, 1985). The bands corresponding to these two proteins were cut out of a preparative 7.5% polyacrylamide gel. The proteins were eluted with PBS, pH 7.0, 0.1% SDS, precipitated with acetone, and resuspended in PBS. This preparation was used for immunization.

**Reduction and Carboxymethylation of Cell Extracts and Flagella**

Cells or isolated flagella were solubilized in 6 M guanidinium chloride, 50 mM morpholino propane sulfonic acid, pH 7.5, and incubated at 37°C for 40 min. Proteins were reduced with 5 mM DTT or with 0.8 mM tributylphosphine (Riegg and Rudinger, 1977). Oxidation of sulfhydryls was catalyzed by 1 mM o-phenanthroline and 0.5 mM CuSO_4. Blocking of free sulfhydryls was performed as described (Kaiser and Murray, 1985). 5 × 10^5 independent recombinant phages were plated into 1.5% agarose gels, and 105 phages of the amplified library were screened, using the polyclonal antiserum as probe. Bound antibodies were detected by incubation with anti-rat IgG conjugated to horseradish peroxidase at a dilution of 1:400 in TBS, pH 7.4, 5% horse serum. Peroxidase activity was visualized with a freshly prepared solution of 0.5 mg/ml diaminobenzidine, 4 mM H_2O_2 in TBS, pH 7.4.

**Antibodies**

Polyclonal antibodies were raised against the 73- and 69-kD proteins in rats. The antiserum was entirely specific as tested by immunoblotting of whole-cell extracts and was used for immunoscreening without further purification. A monoclonal antibody against the PFR proteins was a gift from J.-M. Gallo (University of Poitiers, Poitiers, France) (Gallo and Schrevel, 1985). Peroxidase-conjugated swine antibodies to rat or mouse immunoglobulins (DAKOPATTS, Copenhagen, Denmark) were used to detect bound primary antibodies on Western blots. Goat anti-rat IgGs coupled to 15-nm gold for immunoelectron microscopy were purchased from Janssen Life Science Products (Beerse, Belgium).

**Immunogold Electron Microscopy**

For electron microscopy, Lowicryl-embedded insect and bloodstream form trypanosomes were provided by U.-P. Mothespacher (Tropeninstitut, Basel, Switzerland). Thin sections were mounted on parlodion-carbon-coated nickel grids. After blocking with 5% FCS in 20 mM Tris-HCl, pH 8.2, 150 mM NaCl (TBS), the grids were incubated for 4 h at room temperature with the fusion protein affinity-purified polyclonal anti-PFR antibodies, diluted 1:5 in TBS, 5% FCS. After rinsing in TBS, 5% FCS, the grids were incubated for 2 h with 15-nm gold-labeled goat anti-rat antibody diluted 1:2 in TBS, 5% FCS. Grids were postfixed for 5 min with 1% glutaraldehyde in TBS, rinsed, and contrasted with 2% uranyl acetate and lead citrate. Control incubations received only the second gold-labeled antibody.

**Hybridization Conditions**

DNA-containing filters were prehybridized for 2 h at 37°C with 50 μl/cm² of 0.6 M NaCl, 4 mM EDTA, 0.08 M Tris-HCl, pH 7.8, 0.1% BSA, 0.05% Ficoll, 0.08% polyvinylpyrrolidone, 50% (vol/vol) formamide, 0.2% NaDodSO_4, 0.1% sodium-pyrophosphate, 100 μg/ml deprotonated calf thymus DNA. Subsequent hybridization was carried out with 10^-6 to 10^-7 cpm of 32p-labeled DNA per filter in 30 μl/cm² of the above buffer overnight at 37°C. After hybridization, the filters were washed twice successively in 0.3 M NaCl, 30 mM sodium citrate, pH 7.2 (2× SSC), 0.1% NaDodSO_4 and twice in 0.2× SSC, 0.1% NaDodSO_4. Filters containing DNA hybridized with homologous probes were washed at 60°C, whereas filters containing heterologous probes were washed at room temperature.

**Constructing and Screening of a Genomic DNA Library from T. brucei in Lambda gt II**

High molecular mass DNA from T. brucei stock STIB 366 was prepared as described (Seebbeck et al., 1983). It was sheared by sonication to a length of 0.2–6 kb, treated with T4 DNA polymerase, methylated with Eco RI methylease, and completed with Eco RI linkers before ligation into the unique dephosphorylated Eco RI site of lambda gt 11 (Young and Davis, 1983). The recombinant viral DNA molecules were packaged and plated on Escherichia coli strain Y 1088, giving a total of 6 × 10⁶ recombinants.

Before screening, the library was amplified once on E. coli Y 1088, 6 × 10⁷ phages of the amplified library were screened, using the polyclonal PFR antiserum at a dilution of 1:500 in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS) containing 5% horse serum following published procedures (Young and Davis, 1983). Bound antibodies were detected by incubation with anti-rat IgG conjugated to horseradish peroxidase at a dilution 1:400 in TBS, pH 7.4, 5% horse serum. Peroxidase activity was visualized with a freshly prepared solution of 0.5 mg/ml diaminobenzidine, 4 mM H_2O_2 in TBS, pH 7.4.

**Constructing and Screening of a Genomic DNA Library from T. brucei in Lambda EMBL 4**

A genomic library from T. brucei stock STIB 366 in the lambda vector EMBL 4 (Frischauf et al., 1983) was constructed using published procedures (Kaiser and Murray, 1985). 5 × 10⁶ independent recombinant phages were obtained, and the library was then amplified once on E. coli NM 539. A 180-bp insert of a positive gt 11 phage was isolated and subcloned into a pEP-30 plasmid vector, yielding plasmid pEP72(180). The pEP30 vector is a modification of pGEM-3 vector (Promega Biotec, Madison, WI) containing the single strand replication origin from pEMBL 9 (Dente et al. 1983; Imboden et al., 1987). The resulting pEP72(180) was used to screen the trypanosomal genomic library by DNA hybridization procedures described by Ozaki and Traub-Cseko (1984).
et al., 1985) using lysogenic strains on E. coli Y 1089. The cells were harvested and suspended in 100 mM Tris-HCl, pH 7.4, 2 mM EDTA and frozen down quickly in ethanol-CO₂. A crude lysate was obtained by slow thawing of the cells. As a purification step, an ammonium sulfate precipitation was carried out in 100 mM Tris-HCl, pH 7.4, and the dissolved proteins were precipitated with a 10% final concentration of TCA. The proteins were separated on a preparative 8-15% polyacrylamide gradient gel and transferred onto nitrocellulose. After staining with 0.04% Ponceau S (Fluka AG, Buchs, Switzerland) in 10% acetic acid, the band corresponding to the fusion proteins was excised. The strip was washed twice in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl (TBS), 0.05% Tween 20 for 10 min and incubated overnight at 4°C with the polyclonal PFR antiserum, diluted 1:5 in TBS, 0.05% Tween 20, 3% BSA (Fluka AG). After washing three times for 10 min in TBS, 0.05% Tween 20, elution of the adsorbed antibodies was done essentially as described by Goldstein et al. (1986). The affinity-purified antibodies were tested on Western blots and immunofluorescence for their ability to bind back to PFR proteins. These antibodies were then used for immunogold microscopy.

DNA Sequencing

A 6.4-kb Kpn I-Kpn I fragment containing the genes for PFR1 and PFR2 was subcloned into the Kpn I site of the Bluescript plasmid (Stratagene), yielding plasmid pTBPFRA. Ordered deletions were produced by exonuclease III digestion following the procedures described in the Stratagene manual. Single and double strand sequencing was performed with the Sequenase kit (United States Biochemical Corp., Cleveland, OH) following the dideoxy procedure of Sanger et al. (1977) and according to the instructions of the manufacturer. The sequence was analyzed using the DNAstar software (Madison, WI).

Hybrid Selection and In Vitro Translation

mRNA coding for PFR proteins was hybrid selected from total RNA using pTBPFRA DNA spotted onto nitrocellulose following the procedure of Parnes et al. (1981). The hybrid-selected RNA was subsequently used for in vitro translation in a reticulocyte lysate (New England Nuclear, Boston, MA), using [35S]methionine as labeled amino acid, according to the instructions of the manufacturer. After 1 h of incubation at 37°C, cold methionine was added from a stock solution to a final concentration of 1 mM, and the incubation was continued for another 10 min at 37°C. 15 μl of the translation products was precipitated with 1 ml 10% TCA and washed twice with 5% (vol/vol) 2-mercaptoethanol, and 9.5 M urea. As internal markers, nonlabeled purified 73- and 69-kD PFR proteins (1 μg in 2 μl of the solution described above) were added. This protein mixture was resolved by two-dimensional gel electrophoresis and transferred onto nitrocellulose. The blots were examined by immunostaining with PFR antibody and by autoradiography.

Primer Extension Sequencing

Primers specific for the divergent upstream regions of the mRNAs for PFR-A (5'-CTTTATCTACAAGTGAAT-3') and for PFR-B (5'-TCTCTTAAATTGOGATA-AC-3') were synthesized on a synthesizer (Applied Biosystems, Inc., Foster City, CA) and were purified on NAC-10 minicolumns (Pharmacia Fine Chemicals, Piscataway, NJ). Primer extension was performed essentially as described (Imboden et al., 1986). 100 ng of 5' labeled primer (100,000 cpm) were annealed with 20 μg total trypanosomal RNA in 10 mM Tris-HCl, pH 7.5, 0.25 M KCl, 1 mM EDTA in a final volume of 20 μl at 37°C for 2 h. Then 40 μl of a mixture containing 75 mM Tris-HCl, pH 7.5, 15 mM DTT, 12 mM MgCl₂, 3 μg actinomycin D (Sigma Chemical Co., St. Louis, MO), 12 μl RNasin (Promega Biotech), 20 μl AMV reverse transcriptase (Super RT; Anglian Biotechnology Ltd., Cambridge, UK), and the nucleotide triphosphates was added. Nucleotide triphosphates were present each at 500 μM for the full reaction. For each sequencing reaction, the dideoxy derivative was used at 10 μM and the corresponding deoxy-triphosphate at 20 μM, while the other triphosphates were present each at 40 μM. The reaction mixtures were incubated at 37°C for 1 h. Nucleic acids were then precipitated with ethanol, resuspended in 10 μl loading buffer (96% formamide, 20 mM EDTA, pH 8, 0.1% bromophenol blue, 0.1% xylene cyanol FF), heated for 3 min at 80°C, and analyzed on 6% polyacrylamide gels. An end-labeled Hpa II digest of pBR322 was used as a set size marker.

Other Procedures

One-dimensional gel electrophoresis was performed according to Laemmli (1970). Two-dimensional gel electrophoresis was carried out by the original procedure (O’Farrell, 1975) with the following modifications: (a) the mixture of ampholines used was a 1:1 ratio of pH 4-6/pH 5-8; (b) the prefocusing run was omitted—electrophoresis was done on a mini-gel apparatus at a constant 215 V for 3 h and was then continued for an additional hour at 430 V; and (c) a 10% polyacrylamide gel was used for the second dimension. Transfer of polypeptides to nitrocellulose and immunostaining with poly- and monoclonal antibodies were done as described in Towbin et al. (1979).

Results

The PFR Proteins

The PFR structure of the trypanosomal flagellum consists of a highly ordered, three-dimensional fiber network. It runs parallel to the microtubular axoneme and is closely linked to the latter through a complex network of protein linkers (Fig. 1). The PFR of isolated flagella is highly resistant to extraction by nonionic detergents, low salt buffers, or 1 M NaCl (Schneider et al., 1987), but it can be solubilized with 6 M urea. SDS-gel electrophoresis analysis demonstrated that, in accordance with the literature, the major structural components of the PFR were two proteins with apparent molecular masses of 73 and 69 kD. Attempts to separate the two

Figure 1. (A) Section through a flagella preparation of T. brucei. (B) Cross section through a flagellum. ax, axoneme; pfr, paraflagellar rod. Bars, 0.1 μm.
Figure 2. Migration of PFR protein is dependent on its reduction status. (Lane 1) Whole-cell lysate in 6 M GuCl and 20 mM iodoacetamide; (lane 2) same as lane 1, but oxidized with phenanthroline/copper at 25°C for 30 min; (lane 3) whole-cell lysate in 6 M GuCl; (lane 4) same as lane 3, but incubated at 25°C for 30 min; (lane 5) same as lane 3, but oxidized with phenanthroline/copper at 25°C for 30 min; (lane 6) same as lane 5, but subsequently reduced with 5 mM DTT at 50°C for 30 min; (lane 7) whole-cell lysate in 6 M GuCl and 5 mM DTT. Each slot received the equivalent of 1.3 x 10⁶ cells. Proteins were blotted and visualized by immunostaining with PFR antiserum.

Table I. The Amino Acid Composition of PFR1 and PFR2 as Compared with Intermediate Filaments

| Amino acid | PFR protein* | PFR protein† | Keratin* | Desmin† | Intermediate filament Helix pomatia* |
|------------|--------------|--------------|----------|---------|----------------------------------|
|            | %            | %            | %        | %       |                                 |
| Ala        | 10           | 8.8          | 6.4      | 8.9     | 9.1                              |
| Arg        | 6            | 6.3          | 6.8      | 9.8     | 7.1                              |
| Asn + Asp  | 10           | 9.0          | 10.8     | 8.4     | 9.6                              |
| Cys        | -            | 1.2          | 0.5      | 0.2     | -                                |
| Glu + Glu  | 18           | 20.0         | 16.9     | 20.9    | 17.1                             |
| Gly        | 9            | 3.0          | 9.3      | 4.3     | 7.1                              |
| His        | 2            | 3.0          | 1.0      | 1.6     | 3.0                              |
| Ile        | 4            | 4.8          | 3.7      | 3.9     | 4.9                              |
| Leu        | 8            | 9.8          | 11.2     | 9.3     | 9.0                              |
| Lys        | 8            | 9.8          | 5.4      | 4.6     | 7.0                              |
| Met        | 1            | 3.2          | 3.2      | 2.5     | 1.2                              |
| Phe        | 3            | 2.3          | 2.4      | 3.2     | 2.0                              |
| Pro        | 1            | 1.7          | 0.7      | 1.6     | 2.8                              |
| Ser        | 6            | 4.3          | 8.3      | 6.4     | 8.1                              |
| Thr        | 5            | 3.8          | 4.6      | 5.4     | 5.1                              |
| Trp        | -            | 0.5          | 0.5      | 0.2     | -                                |
| Tyr        | 1            | 2.2          | 2.9      | 2.5     | 3.1                              |
| Val        | 6            | 6.0          | 5.4      | 6.2     | 3.9                              |

Values are presented as percentages.
* Biochemical determination (intermediate filament Helix pomatia from Bartnik et al. [1985]).
† Calculated from the deduced protein sequence (vimentin from Hanukoglu and Fuchs [1982] and desmin from Geisler et al. [1982]).

The amino acid composition of purified PFR protein was found to bear similarity to that of intermediate filaments (Table I), suggesting that the proteins might contain a large proportion of helical structure. In agreement with this assumption, limited digestions of PFR protein with trypsin or chymotrypsin produce a protease-resistant fragment of 65 kD, suggesting the presence of a tightly organized, protease-resistant core flanked by more loosely structured, protease-sensitive NH₂- and COOH-terminal domains (not shown).

The Journal of Cell Biology, Volume 109, 1989 1698
between different trypanosomal subgenera and species. All species of the subgenus *Trypanozoon*, (*T. b. brucei*, *T. b. rhodesiense*, and *T. b. gambiense*) react equally well with the polyclonal antiserum (Fig. 3, lanes 1–3). Similarly, representatives of the subgenus *Nannomonas* (*T. congolense*, stock K44, and *T. simiae*; lanes 4 and 7) also react strongly. In contrast to these salivarian trypanosomes, the stercorarian species *T. cruzi* (*Schizotrypanum*) (lane 5) and *T. rangeli* (*Herpetosoma*) (lane 6) exhibit a much weaker reaction. In all these species, the proteins detected by the antibodies migrate in the same molecular mass range as those of *T. brucei*. This is in agreement with a previous study (Gallo and Schrevel, 1985) that described two paraflagellar proteins in *T. brucei* of 75 and 72 kD. The same pattern was also obtained when the bloodstream forms of *T. brucei* were stained with the same antibody (not shown). The apparent molecular masses of the PFR proteins of the more distantly related kinetoplastids *C. fasciculata*, *L. major*, and *L. donovani* (lanes 8, 10, and 11) are slightly different from those of *T. brucei*, in agreement with Russell et al. (1983) who determined appar-

Figure 3. Sections of Lowicryl-embedded procyclic trypanosomes after immunogold staining by anti-PFR antibody affinity purified on the β-galactosidase-PFR fusion protein of TbPFR 72. Bar, 0.1 μm.
ent molecular masses of 76 and 68 kD for the corresponding proteins in *C. fasciculata*.

In contrast to *C. fasciculata*, only a very weak reaction was observed with *C. deanei* (lane 9), and no reaction was detectable in *C. oncopelti* (not shown). The absence of PFR staining in these species is in agreement with, and extends, the earlier observations of Freymuller and Camargo (1981) who have shown that neither of these species contains a PFR structure that is detectable by electron microscopy. The presence of traces of PFR protein in *C. deanei* may suggest that this species still contains a low level of PFR protein, though it is apparently not organized into a discernible PFR structure.

Among the nonkinetoplastid organisms tested, a very weakly positive signal was obtained with *E. gracilis*. According to Hyams (1982), *E. gracilis* contains a rod-like structure within its flagellum which is made up predominantly of two polypeptides of 80 and 69 kD. The PFR antibody in fact does stain two polypeptides at the appropriate molecular mass range (lane 16). Besides *Euglena*, none of the other protozoa tested (*G. lamblia, Tet. pyriformis, D. discoideum, and C. reinhardtii*) reacted with the PFR antibody.

**The PFR Protein Is Coded for by Two Genes That Are Tandemly Linked**

The PFR antiserum was used for screening a genomic lambda phage gt11 expression library of *T. brucei*. The 180-bp insert from a recombinant phage (λTbPFR72) isolated from this screening was subcloned into the pEP 30 vector.

![Physical map of the PFR locus](image)

*(A)* A partial restriction map of the seven overlapping bacteriophage recombinants (AEML4Tb A-G) covering a total of 23 kb. *(B)* A 6.4-kb Kpn I fragment hybridizes with the coding sequence of pEP72(180), and the 5-kb Hind II–Kpn I fragment has been sequenced. The positions of the two coding regions are indicated by solid boxes. The direction of transcription is from left to right. *H*, Hind III; *K*, Kpn I; *R*, Eco RI; *C*, Cla I; *I*, Hind II; *P*, Pst I; *V*, Pvu II.
and the resulting pEP72(180) was then used to screen a genomic DNA library in λ.EMBL4. Seven individual phages were purified, and restriction analysis of their isolated DNA demonstrated that these seven recombinants all contained overlapping DNA regions from the same genetic locus, covering a total of 23 kb. Within the recombinant EMBL phage DNA, a 6.4-kb Kpn I fragment was identified that carried PFR coding sequence. This Kpn I fragment was subcloned into a Bluescript vector, and the resulting plasmid pTbPFR-A was analyzed by restriction mapping and hybridization. The detailed physical map of the PFR locus is presented in Fig. 5. The locus contains two closely spaced regions that both code for PFR. A degree of similarity between the two is indicated by a number of restriction sites that are identical between the two regions.

The total number of PFR loci in the genome of *T. brucei* was determined by restriction enzyme analysis of genomic DNA. Hybridization of restriction digest of genomic DNA with the PFR-specific probe pTbPFR-A (see Fig. 5) revealed a band pattern (Fig. 6A) that is fully compatible with the restriction map of the PFR locus given in Fig. 5. Thus, no PFR genes are located elsewhere in the trypanosomal genome. This conclusion was further corroborated by titration hybridization of genomic DNA digests. Genomic DNA (2 and 4 μg) was doubly digested with Cla I and Pvu II. The isolated 2.2-kb Cla I probe, digested with Pvu II, was hybridized to a Southern blot of *T. brucei* genomic DNA (2 and 4 μg) restricted with Cla I and Pvu II. Carefully calibrated amounts of the purified 2.2-kb Cla I fragment, restricted with Pvu II, are present in lanes 1-7 (lanes 1-5) 0, 110, 220, 550, and 770 pg, respectively; (lanes 6 and 7) 1.1 and 2.2 ng, respectively. Lanes 1-7 represent, respectively, 0, 1, 2, 5, 7, 10, and 20 copies of the 2.2-kb fragment relative to the *T. brucei* genome content in 2 μg DNA.

**Figure 6.** (A) Genomic hybridization. Southern blot of genomic DNA from *T. brucei* (2 μg/ lane) digested with restriction enzymes and hybridized with labeled pTbPFR-A. (Lane 1) Kpn I; (lane 2) Cla I; (lane 3) Cla I-Pvu II; (lane 4) Kpn I-Cla I. The arrowhead indicates a barely visible band in lane 4. (B) Copy number of the 0.95-kb Cla I-Pvu II fragment of the coding regions of genes A and B. The isolated 2.2-kb Cla I probe, digested with Pvu II, was hybridized to a Southern blot of *T. brucei* genomic DNA (2 and 4 μg) restricted with Cla I and Pvu II. Carefully calibrated amounts of the purified 2.2-kb Cla I fragment, restricted with Pvu II, are present in lanes 1-7 (lanes 1-5) 0, 110, 220, 550, and 770 pg, respectively; (lanes 6 and 7) 1.1 and 2.2 ng, respectively. Lanes 1-7 represent, respectively, 0, 1, 2, 5, 7, 10, and 20 copies of the 2.2-kb fragment relative to the *T. brucei* genome content in 2 μg DNA.

**DNA Sequence and the Deduced Amino Acid Sequence**

From the physical map of the PFR locus given in Fig. 5, the 5-kb Hind II-Kpn I fragment was selected for sequencing. Its complete nucleotide sequence is given in Fig. 7.

The analysis of the sequence reveals the presence of two closely linked, tandemly repeated open reading frames (PFR-A and PFR-B) of identical length (1,800 bp) and of identical nucleotide sequence, each coding for the identical protein of 600 amino acids. Outside these open reading frames, the similarity between the two genes rapidly degenerates. The nucleotide sequence of the PFR gene fragment of the XbPFR72 phage originally identified by the immunoscreening is represented by nucleotides 1,449-1,619 and 3,603-3,773 within the coding areas of PFR-A and PFR-B in Fig. 7.

The calculated molecular mass of the protein coded for by PFR-A and PFR-B (69.9 kD) and its calculated isoelectric point (5.87) correspond well with the values determined experimentally for nonreduced PFR protein (see above; Rindbacher, L., and T. Seebeck, unpublished observations). Similarly, the calculated amino acid composition fully agrees with the values determined biochemically (Table I). Codon usage in the PFR open reading frames is similar to that of other trypanosomal genes, such as the tubulin genes (Kimmel et al., 1985) or fructose bisphosphate aldolase (Clayton, 1985). The trypanosomal codon usage differs from that of most eukaryotes by a strong preference for the argi-
Genes PFR A+B

The Journal of Cell Biology, Volume 109, 1989 1702
The Journal of Cell Biology, Volume 109, 1989

Fig. 7
nine codons CGC and CGT and the avoidance of AUA and UUA as codons for isoleucin and leucine, respectively.

Secondary structure prediction indicates that the protein coded for by PFR-A and PFR-B may assume a mostly helical conformation throughout its entire length (>80% helicity predicted by the method of Gamier et al., 1978). However, no evidence was found throughout the sequence for a repeating heptad motif which is characteristic for the helical domains of many filamentous proteins and which enables them to form coiled coil structures due to hydrophobic interactions between opposing amino acids along the helix surface (McLachlan, 1984). The amino acid distribution along most of the helical domains of PFR would allow the formation of ion pairs along the helix at a high frequency, which would significantly contribute to a stabilization of this structure (Sundaralingam et al., 1985). This observation further suggests that the PFR protein is in fact largely helical in its native configuration. Amino acids 335-355 may form a calmodulin-binding site (Ericksson-Viitanen and DeGrado, 1987). Similarity searches of the protein sequence library (PIR release 18) revealed that the COOH terminus of PFR (amino acids 587-600; SQQYRGRTMPQITQ) is closely similar to a highly conserved sequence found in all β-tubulins around tyrosine residue 281. In β-tubulin, this region may be involved in dimer recognition and polymerization (Rudolph et al., 1987; Fridovitch-Keil et al., 1987). Its function on PFR remains to be elucidated.

A nucleotide sequence survey of the GenBank/EMBL DNA sequence libraries revealed identities of 77 and 43 bp for PFR-A and PFR-B, respectively, with a previously published partial cDNA sequence from T. brucei (pSLcl; Parsons et al., 1984). The pSLcl sequence was shown to represent the 5' terminus of a then unidentified mRNA carrying the mini-exon sequence. The 77 nucleotides after the mini-exon, whose sequence had been determined by Parsons et al., are almost identical to nucleotides 499-577 of the PFR-A sequence (including the first eight codons of PFR-A). The only difference between the two sequences is at position 571.
(PFR-A) and 2,725 (PFR-B), where Parson's sequence contains a G, while the sequence presented in Fig. 7 contains an A. The shorter extent of similarity between pSLcl and PFR-B is due to the degeneration of similarity between PFR-A and PFR-B upstream of the coding sequence. Thus, the similarity between pSLcl and the PFR locus indicates that pSLcl represents the mRNA transcribed from PFR-A.

**PFR-A and PFR-B Code for Distinct mRNAs**

To identify potential mRNAs derived from PFR-A and PFR-B, total trypanosomal RNA was analyzed by primer extension sequencing. The two primers were selected to represent the divergent upstream regions of each gene to allow the identification of transcripts from each gene (Fig. 8). The results summarized in Fig. 8 indicate that trypanosomes contain stable mRNAs derived from both genes, PFR-A and PFR-B. Both PFR mRNA species carry the mini-exon sequence at their 5' termini. The sequence of PFR-A mRNA is identical to the one reported earlier as the 5' terminus of an unidentified mRNA (Parsons et al., 1984). The nucleotide sequences of the two mRNAs diverged along most of the 5' leader, while the sequences of the last 19 nucleotides immediately upstream of the initiator AUG, as well as the entire coding sequence, are identical. While the length of the two 5' leader sequences differs by only 11 nucleotides, Northern blotting experiments (not shown) indicate that also the entire length of both mRNAs must be very similar.

**PFR Protein Synthesized In Vitro Migrates as a Doublet in Reduced Form**

Much published evidence, as well as our own experiments (e.g. Fig. 4), suggested that the PFR protein migrates as two distinct bands in SDS-gel electrophoresis. However, the evidence presented in Fig. 2 strongly indicates that the more slowly migrating of the two bands represents a reduced derivative of the single PFR protein. This conclusion was now reinvestigated, and confirmed, with PFR protein synthesized in vitro. PFR mRNA was isolated by preparative hybridization of total trypanosomal RNA to filter-bound pTbPFRA DNA. The hybrid-selected mRNA was released from the filters and translated in a rabbit reticulocyte lysate. Total translation products were then solubilized in reducing sample buffer, mixed with purified, unlabeled PFR proteins as internal markers, and fractionated by two-dimensional gel electrophoresis. After transfer to nitrocellulose filters, the marker PFR proteins were visualized by immunostaining, and the filter was then exposed for autoradiographic detection of the PFR proteins synthesized in vitro. The results given in Fig. 9 demonstrate that in vitro synthesis results in two radioactive spots that comigrate with the two spots formed by the authentic marker PFR protein. Thus, PFR protein synthesized in vitro from hybrid-selected mRNA displays the same migration behavior as does the PFR protein extracted from trypanosome cells. The experiment illustrated in Fig. 9 thus confirms that the two PFR protein species observed under reducing conditions represent two conformers of a single polypeptide chain, which is coded for by the genetic locus whose detailed structure has been reported in this study.

### Discussion

The PFR structure is a highly ordered three-dimensional network of fibrous proteins which is a specific structural component of the flagella of trypanosomatids and euglenoids. This common structure may reflect a common pathway of evolution of these two groups of flagellates, which are currently thought to have split from the mainstream of eukary-
Figure 9. mRNA hybrid selected on pTbPFR-A and translated in vitro gives rise to two proteins that comigrate with authentic PFR in twodimensional gel electrophoresis. (A) Immunoblot of the 35S-labeled in vitro translation products mixed with purified, unlabeled PFR protein (1 μg). (B) Autoradiograph of the same filter. Gels are represented with the acidic end to the left.

otic evolution very early on (Sogin et al., 1986). Though the structural details of the PFR differ between the two groups (Hyams, 1982), immunological evidence has been presented that their major proteins may be very similar. The present study represents an analysis of the major structural protein (PFR protein) of the PFR of T. brucei.

Antibodies raised against PFR protein were used to isolate the corresponding genes from an expression library. The PFR protein is coded for by a single locus of the trypanosomal genome. DNA sequence analysis of this locus revealed the presence of two closely spaced open reading frames of 1,800 nucleotides length, each, for PFR-A and PFR-B. The nucleotide sequence of both reading frames is identical and, thus, both of them code for an identical protein of 600 amino acids. Outside the coding regions, the similarity between the two genes rapidly degenerates. Upstream of the initial AUG codon, the sequences of PFR-A and PFR-B are identical for an additional 19 bp, but then become dissimilar. No similarity whatsoever is found after the TAG termination codons.

A survey of DNA sequence libraries revealed that a previously published 77-bp sequence from the 5' terminus of a then unidentified mini-exon mRNA (cDNA clone pSLc1; Parsons et al., 1984) is identical to the 5' terminus of PFR-A, including the first eight codons of this gene.

Primer extension sequencing of the PFR mRNAs revealed that stable mRNAs are generated both from the PFR-A and PFR-B genes. The mRNAs contain the mini-exon sequence at the 5' terminus of a then unidentified mini-exon mRNA (cDNA clone pSLc1; Parsons et al., 1984) is identical to the 5' terminus of PFR-A, including the first eight codons of this gene.

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The amino acid composition of PFR calculated from the derived amino acid sequence corresponds very well with that determined biochemically for isolated PFR protein. Also, the calculated molecular mass and isoelectric point are very similar to the values determined experimentally. Secondary structure prediction of the protein indicates a very high content of α-helix. This is in good agreement with the marked resistance of the major part of the protein to protease digestion. The PFR protein bears remarkable similarity to intermediate filament proteins in that (a) it is insoluble under a variety of salt and detergent conditions; (b) its overall amino acid composition is similar; and (c) it has a high content of helical regions. In contrast to intermediate filament proteins, no evidence was found in the sequence of PFR protein for a heptadic arrangement of hydrophobic amino acids, which is characteristic for many helical proteins assuming a coiled coil configuration (McLachlan, 1984). A PFR protein sequence library search indicated that the COOH terminus of PFR is similar to a highly conserved region found in all β-tubulins around tyrosine residue 281. While in β-tubulin this region may be involved in polymerization, its functional significance in the PFR protein remains to be established.

The PFR gene locus codes for a single polypeptide of 600 amino acids, corresponding to a calculated molecular mass of 69 kD. This is at variance with the observation in cell extracts of two distinct, approximately equimolar, bands that migrate on denaturing gels with apparent molecular masses of 73 and 69 kD. This migration is unaltered after exposure to reducing conditions, such as incubation with DTT or tributylphosphine, whereas oxidation leads to a single band migrating at an apparent molecular mass of 69 kD. Translation of hybrid-selected PFR mRNA in vitro similarly generates two polypeptides with different migration properties. Upon two-dimensional gel electrophoresis, the translation products comigrate with authentic PFR protein isolated from cells, forming two distinct spots. This migration pattern of PFR protein synthesized in vitro confirms that the two spots (or bands in one-dimensional electrophoresis) observed by many workers (for review see Cachon et al., 1988) represent in fact two conformations of a single polypeptide chain. In addition, the in vitro translation experiments serve to rule out the possible existence of a second set of PFR genes that might potentially code for the 73-kD protein, but whose nucleotide sequence might have diverged so far as to be no more detectable by hybridization with the PFR gene described in this study. Furthermore, a possible generation of the 73-kD PFR protein species from the 69-kD variety through posttranslational modification is also rendered unlikely by
the results of in vitro translation. In summary, the 73- and 69-kD variants of PFR most likely represent different conformations of the single polypeptide coded for by the PFR locus.

The PFR protein represents a novel type of cytoskeletal protein that is restricted to the trypanosomatids and, most likely, the Euglenoids. Considering the fact that the trypanosomatids are parasitic organisms, this parasite-specific structure might not only be of interest in terms of cellular architecture and function, but it also may represent a potential, highly parasite-specific target for trypanocidal drugs.

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