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Differential Accumulation of a Protein Kinase Homolog in *Trypanosoma brucei*[1]

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Abstract Using degenerate oligonucleotide primers derived from conserved regions in the catalytic domains of protein kinases, we have identified transcripts of the protein kinase families in *Trypanosoma brucei* by the polymerase chain reaction technique. From the cDNAs synthesized from poly(A)$^+$ RNA purified from the bloodstream form of the pathogen, we have obtained seven distinct partial cDNA sequences. Deduced amino acid sequences of these seven clones contain conserved regions characteristic of catalytic domains of eukaryotic protein serine/threonine kinases. DNA gel blots showed that one of the clones, TbPK-A4 is most likely a member of a subfamily in the protein kinase gene family, whereas the other six are probably each encoded by a single gene in the genome of *T. brucei*. The full-length cDNA of TbPK-A1 was cloned, sequenced, and found to encode an open reading frame of 350 amino acid residues. Its gene (designated *KFR1*) demonstrated high sequence similarity to *KSS1* and *FUS3* from *Saccharomyces cerevisiae* and rat MAP kinase at the amino acid level. There are a 3- to 4-fold higher level of *KFR1* transcript and a 2-fold increase of *KFR1* protein in the bloodstream form when compared with the insect form of *T. brucei*. This preferential expression of *KFR1* in the bloodstream form of *T. brucei* may play a role in controlling the cell cycle and thus the growth rate of the organism.

Key words: cell proliferation, *FUS3, KSS1, KFR1,* MAP kinase, trypanosome

Phosphorylation of specific proteins by protein kinases has been recognized as a major strategy for regulating protein and enzyme activities in response to the transduction of environmental, developmental, and metabolic signals in mammalian cells as well as in lower-order eukaryotes [Krebs, 1985; Edelman et al., 1987; Soderling, 1990; Ullrich and Schlessinger, 1990]. Various protein kinases respond to different signals and serve in different regulatory roles in the cell. There are few, if any, physiological processes in eukaryotes that are not dependent on protein phosphorylation. In spite of the tremendous diversity of protein kinases, they all share a common catalytic domain which typically extends over 250–300 amino acid residues. The amino acid sequences in the catalytic domain of these kinases are well conserved among animals, plants, and yeasts [Hanks et al., 1988]. Thus, degenerate oligonucleotides encoding the conserved regions have been effectively used as hybridization probes to identify members of protein kinase families from mammalians [Hanks, 1987], plants [Lawton et al., 1989], and yeast [Levin et al., 1987]. Alternatively, protein kinase genes can be amplified by polymerase chain reactions (PCR) using degenerate oligonucleotides corresponding to the conserved regions as primers [Feng and Kung, 1991].

Trypanosoma brucei is a parasitic protozoan causing African sleeping sickness in man and nagana in cattle. This extracellular parasite passes through several morphologically and physiologically distinguishable stages during its development in the mammalian hosts (bloodstream form) and in the tsetse fly vector (procyclic form) [Vickerman, 1986]. The specific mechanisms that trigger its proliferation and differentiation, however, are not yet understood. Recently, protein kinase activities have been demonstrated in the trypanosome [Aboagye-Kwarteng et al., 1991; Wheeler-Alm and Shapiro, 1992]. Parsons and coworkers detected protein tyrosine kinase and serine/threonine

[1]The nucleotide sequences in the report will appear in the GenBank under the accession numbers L10991 through L10998 for TbPK-A1, TbPK-A2, TbPK-A4, TbPK-A5, TbPK-B1, TbPK-B2, KFR1, and TbPK-A3, respectively.

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kinase activities and demonstrated different phosphorylation patterns during the life cycle of *T. brucei,* suggesting that protein kinases play important roles in the parasite during different stages of its development [Parsons et al., 1991, 1993]. More recently, Gale and Parsons [1993] reported a constitutively expressed protein kinase gene in *T. brucei* which is related to Nek1 and NIMA. Although little information is available concerning specific functions of individual trypanosome protein kinases, it is reasonable to assume that such kinases act as key enzymes in the regulation of cell proliferation and differentiation of *T. brucei.* Molecular cloning of *T. brucei* protein kinases could initiate a molecular genetic approach to understand functions of specific protein kinases in the parasite. With this in mind, we used PCR to identify putative protein kinase genes in *T. brucei* and analyze their expression at different stages of its life cycle.

MATERIALS AND METHODS

Trypanosomes

The procyclic form of *Trypanosoma brucei brucei* TREU667 was cultured in Cunningham medium [Cunningham, 1977] supplemented with 10% fetal calf serum (Gemini Bio-Products, Inc., Calabasas, CA). The bloodstream form of *T. brucei* was propagated in mice as described previously [Bass and Wang, 1991].

PCR Amplification

Poly(A)$^+$ RNA was isolated from *T. brucei* using the Fast-track mRNA isolation system from Invitrogen (San Diego, CA), according to the manufacturer's instructions. The first strand cDNA was made with cDNA cycle kit of Invitrogen. Briefly, the poly(A)$^+$ RNA was primed with oligo(dT)$_{12-18}$ in the presence of MeHgOH and placental RNase inhibitor. The single-stranded cDNAs were then used as templates for the PCR amplification in a 50 μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM each dNTP, 1 μM each primer, 0.001% gelatin, and 2.5 units of Ampli Taq DNA polymerase (Perkin-Elmer, Norwalk, CT) with the temperature cycle of 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min. The nucleotide sequences of the primers are as follows: Primer 1: 5′-CG(ACGT)GA(CT)CT(ACGT)AA(AG)C(CT)(ACGT)GA(ACGT)AA(CT); Primer A: 5′-A(CT)(ACGT)CC(AG)(AT)A(ACGT)G(AC)CCA(ACGT)AC(AG)TC; and Primer B: 5′-(CT)TC(ACGT)GG(ACGT)GC(ACGT)A(CGT)(AG)(AT)A(ACGT)TC(ACGT)(AG)G(ACGT)G(AT)(ACGT)(AC)C. The amplified products were fractionated on a low-melting-point agarose gel. DNA fragments corresponding to 100 bp to 400 bp in size were recovered from the gel and ligated to the *p*CR1000 vector (Invitrogen). Alternatively, the amplified PCR products were directly ligated to the *p*CR1000 vector. The sequences of the inserts were determined by dideoxynucleotide chain termination method [Sanger et al., 1977] using Sequenase 2.0 (US Biochemical, Cleveland, OH). The double-stranded DNA sequencing results were further confirmed by single-stranded DNA sequencing after excising and ligating the PCR inserts into M13mp18 and M13mp19 vectors.

Southern, Northern, and Slot Blot Hybridizations

Total cellular DNA was isolated from the procyclic form of *T. brucei* as follows. Cells were lysed in 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 100 mM EDTA, 20 μg/ml RNase A, 0.5% SDS, and 0.1 mg/ml proteinase K at 50°C for 3 h. The lysate was extracted with phenol, phenol-chloroform (50/50; v/v), and chloroform sequentially. After precipitation with sodium acetate and ethanol, the DNA mass was taken out with a glass rod, rinsed in 70% ethanol and resuspended in TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA). The DNA was then digested with restriction endonucleases and fractionated on a 0.9% agarose gel and Southern-blotted onto a nitrocellulose membrane as described [Sambrook et al., 1989]. The Southern-blotted membranes were probed with the cloned PCR inserts individually at 42°C for 24 h in a solution containing 50% (v/v) formamide, 3% (w/v) nonfat dry milk, 5 × SSC (1 × SSC contains 150 mM NaCl and 15 mM sodium citrate, pH 7.4), 25 mM phosphate buffer (pH 7.0), and 0.1 mg/ml salmon sperm DNA. The DNA probes were radio-labeled with ^{32}P-dATP using random primers DNA labeling system [Sambrook et al., 1989]. The hybridized membrane was washed once in 2 × SSC-0.1% SDS, 0.5 × SSC-0.1% SDS at room temperature, and twice in 0.1 × SSC-0.1% SDS at 60°C. For Northern hybridization, 1 μg poly(A)$^+$ RNA was denatured with glyoxal, fractionated on a 1.1% agarose gel and transferred onto a nitrocellulose membrane as described [Sambrook et al., 1989]. For the slot blot, 0.75 μg poly(A)$^+$ RNA of *T. brucei* and 5 μg of yeast tRNA (Gibco-BRL, Gaithersberg, MD) were denatured with glyoxal

and blotted onto a nitrocellulose membrane as described [Sambrook et al., 1989]. The hybridization and washing conditions for the RNA blots and slot blots were as described for the Southern blots, except that the 3% nonfat milk was replaced by 0.1% Ficoll, 0.1% polyvinylpyrrolidone, and 0.1% bovine serum albumin in the Northern and slot blot hybridization. The washed membranes were exposed to Kodak XAR-5 film at −70°C with intensifying screens. To reprobe the Northern and slot blots, the previously hybridized blots were washed in 0.1 × SSC-0.1% SDS at 100°C for 5 min and hybridized with another probe as described above. The Northern and slot blot hybridization results were analyzed using a laser densitometer (Model 2202 Ultroscan, LKB). Each Northern blot hybridization was carried out two or more times to confirm the results.

cDNA Library Construction and Screening

A cDNA library of *T. brucei* was constructed in vector λZAP II (Stratagene, La Jolla, CA) from the poly(A)$^+$ RNA of *T. brucei* procyclic form using the Superscript system (Gibco-BRL). The library was screened and plasmids of the positive clones were excised according to the instructions of the manufacturer (Stratagene). Restriction maps of the positive clones were analyzed. The inserts were subcloned into M13 vectors and their nucleotide sequences were determined by dideoxynucleotide chain termination method as above [Sanger et al., 1977]. Both strands of the cDNA inserts were sequenced.

Antibody Production and Immunostaining

Three peptides were synthesized on a peptide synthesizer (Applied Biosystems, Inc.) at the Institute of Molecular Biology, Academia Sinica, Taipei. The synthesized peptides were conjugated to keyhole limpet hemocyanin [Harlow and Lane, 1988] and used to immunize rabbits. The amino acid sequences of these peptides are as follows: TbKH1: NH_2-DQYRSALYDIIGNRRKQK-NH_2 (corresponding to residues 330–347 at the C-terminus of *KFR1* protein); TbKH2: NH_2-VSFSIDGRIEQRYK-NH_2 (residues 2–15 at the N-terminus); and TbKH3: NH_2-YFAPFTSPSDFEELDK-NH_2 (residues 298–313). Titers of antisera were monitored by ELISA assay against unconjugated peptides. After the third monthly boost, all three antisera showed high titers in ELISA, each giving positive results beyond 20,000-fold dilution. The antisera collected 105 days after initial immunization were used for immunostaining.

T. brucei cells were lysed in lysis buffer [50 mM Tris-HCl, pH 6.8, 0.5% SDS, 1 mM phenylmethane sulfonylfluoride (PMSF)] at 100°C for 5 min. The lysate was centrifuged at 16,000*g* at 4°C for 10 min. The supernatant was fractionated on a 12.5% SDS-PAGE. The separated proteins were electroblotted onto Immobilon PVDF membrane (Millipore, Bedford, MA). The blotted membrane was incubated in 3% bovine serum albumin (BSA) in TBS-TN [20 mM Tris-HCl, pH 7.4, 0.9% (w/v) NaCl, 0.2% (v/v) Tween-20, 0.3% (v/v) NP-40] for 0.5 h, followed by probing with the antisera against TbKH's (1:4,000 dilution) in TBS-TN with 3% BSA for 1 h. Mouse monoclonal antibody against chicken α-tubulin (Sigma Chemical Co., St. Louis, MO) was used at 1:500 dilution. After incubation with goat anti-rabbit IgG-horse radish peroxidase (HRP) conjugate or goat anti-mouse IgG-HRP, the immune reactions were detected with the ECL chemiluminescence system (Amersham, Arlington Heights, IL). The immunostaining results were also analyzed using a laser densitometer (Model 2202 Ultroscan, LKB). Protein concentrations were measured with Bio-Rad (Richmond, CA) protein assay reagent using bovine IgG as standard.

RESULTS

Identification of Genes Encoding Protein Kinases in *T. brucei*

All known protein kinases share sequence similarities in their catalytic domains and eleven regions of the domains are highly conserved [Hanks et al., 1988]. We therefore synthesized mixed degenerate oligonucleotides encoding the highly conserved regions VI (primer 1), VIII (primer B), and IX (primer A) within the catalytic domains of protein serine/threonine kinases [Hanks et al., 1988]. These oligonucleotide primers, shown in Figure 1A, were used for PCR amplification of cDNAs encoding the protein kinase homologs in *T. brucei*. The first-strand cDNA was synthesized from the poly(A)$^+$ RNA purified from the bloodstream form of *T. brucei*. Two pairs of primers, primer 1 with primer A and primer 1 with primer B, were used (Fig. 1A). From 112 PCR clones with inserts sequenced partially, a total of eighteen clones was found containing the anticipated conserved sequences of protein kinases. There are seven distinct sequences among these eighteen clones.

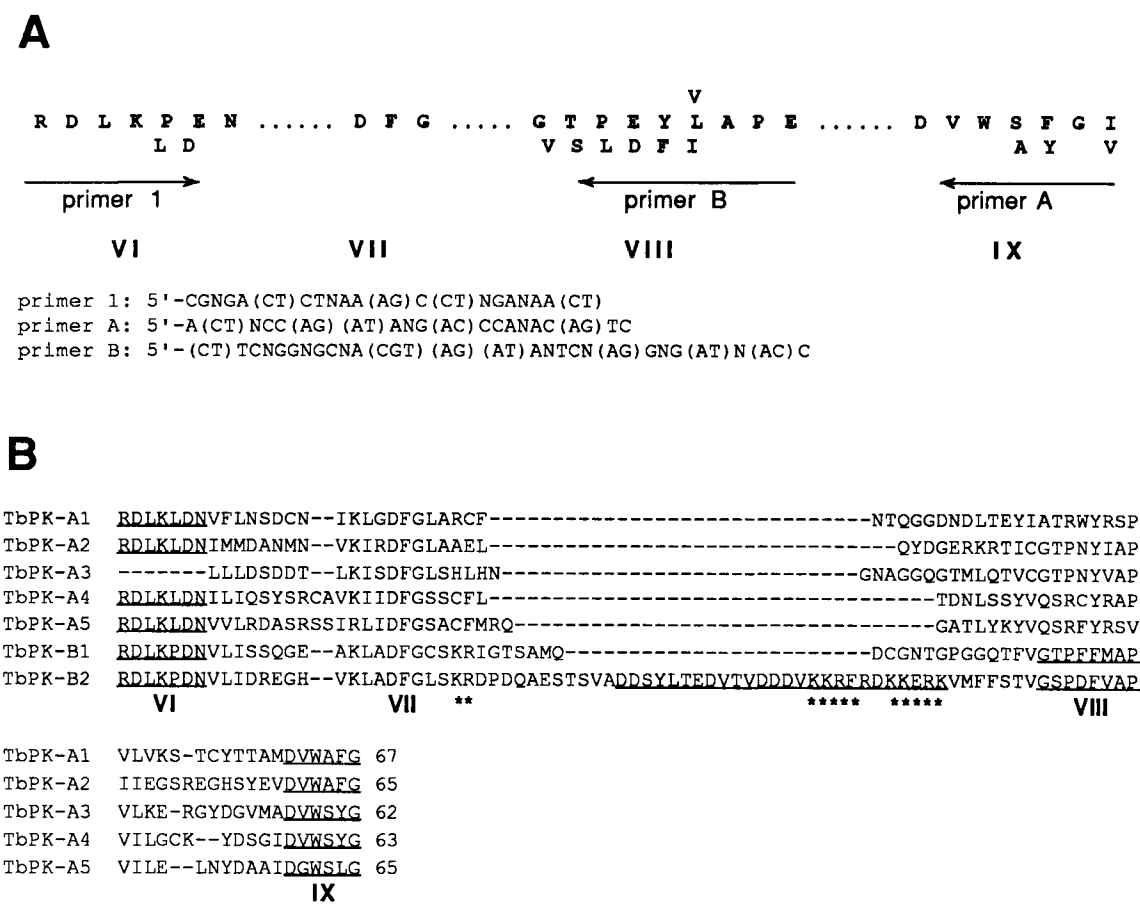

Fig. 1. Polymerase chain reactions. **A:** Amino acid sequences of four conserved regions of protein kinases (indicated in Roman numerals) [Hanks et al., 1988]. Oligonucleotides primers corresponding to these regions (primer 1 with primer A, and primer 1 with primer B) were used for amplification of protein kinases from cDNA of *Trypanosoma brucei* by PCR. The nucleotide sequences of these primers are shown beneath, where "N" represents mixture of A, C, G, and T. **B:** Alignment of deduced amino acid sequences of seven distinct cDNA clones of *T. brucei* protein kinases (TbPK series) amplified by PCR. Dashes (-) represent gaps introduced to optimize the alignment among the TbPK series. Sequences corresponding to the primers are underlined. A very hydrophilic fragment between region VII and VIII of TbPK-B2 is also underlined, whereas the sequences which match the nuclear targeting signal are indicated by asterisks.

These seven clones, designated TbPK (*T. brucei* Protein Kinase) -A1, -A2, -A3, -A4, -A5, -B1, and -B2, were sequenced. Each was found to contain an open reading frame, and their deduced amino acid sequences are aligned in Figure 1B. The seven clones all contain the internal conserved regions of protein kinases. The TbPK-As contain the regions VII and VIII, and the TbPK-Bs contain the region VII (Fig. 1), indicating that all these TbPK clones indeed encode the conserved regions of putative protein kinases. For reasons currently unknown, the primer 1 sequence is not present in the clone TbPK-A3.

Sequence similarity analyses of the seven cDNA clones indicated that TbPK-A4 and TbPK-A5 are closely related, with a 47% amino acid identity excluding the primer regions, while the others share less than 36% identity with one another. We also compared the amino acid sequences of these TbPKs to the GenBank, PIR, and Swiss-Prot databases. The results are shown in Table I.

Genomic Organization of the Putative Protein Kinases in *T. brucei*

To confirm that the seven putative protein kinase clones are derived from the trypanosome, rather than from the host (mouse) blood, we carried out Southern hybridizations. Cellular DNA from the procyclic form of *T. brucei* was restriction-digested with EcoR I and Hind III, and probed with the cDNA inserts of TbPKs. The results are shown in Figure 2. All of the TbPK cDNA probes hybridized strongly with

TABLE I. Amino Acid Sequence Similarity of TbPKs to Their Homologous Protein Kinases in the Databases

	Homologous protein kinases				
TbPKs	Name	Putative functions	Identity (%)	Organisms	References
TbPK-A1	KSS1	Cell growth	49	*S. cerevisiae*	Courchesne et al., 1989
	FUS3	Cell mating	49	*S. cerevisiae*	Elion et al., 1990
	MAPK	Cell growth	48	Rat	Boulton et al., 1990
	CDC28	Cell cycle control	49	*S. cerevisiae*	Lorincz and Reed, 1984
TbPK-A2	Polo	Mitosis	58	*Drosophila*	Llamazares et al., 1991
	PKC	Muti-functional	56	Rat	Ono et al., 1988a, 1988b
TbPK-A3	SNF1	Glucose repression	49	*S. cerevisiae*	Celenza and Carlson, 1986
	AKin10	Carbon metabolism	50	*Arabidopsis*	Le Guen et al., 1992
	MLCK	Myosin-light-chain phosphorylation	49	*Dictyostelium*	Tan and Spudich, 1991
TbPK-A4	YAK1	Unknown	53	*S. cerevisiae*	Garret and Broach, 1989
	MAPK	Cell growth	44	Rat	Boulton et al., 1990
TbPK-A5	YAK1	Unknown	48	*S. cerevisiae*	Garret and Broach, 1989
TbPK-B1	PKC	Unknown	47	*Drosophila*	Schaeffer et al., 1989
	NIMA	Mitotic regulation	42	*Aspergillus*	Osmani et al., 1988
TbPK-B2	COT-1	Hyphal elongation	34	*Neurospora*	Yarden et al., 1992

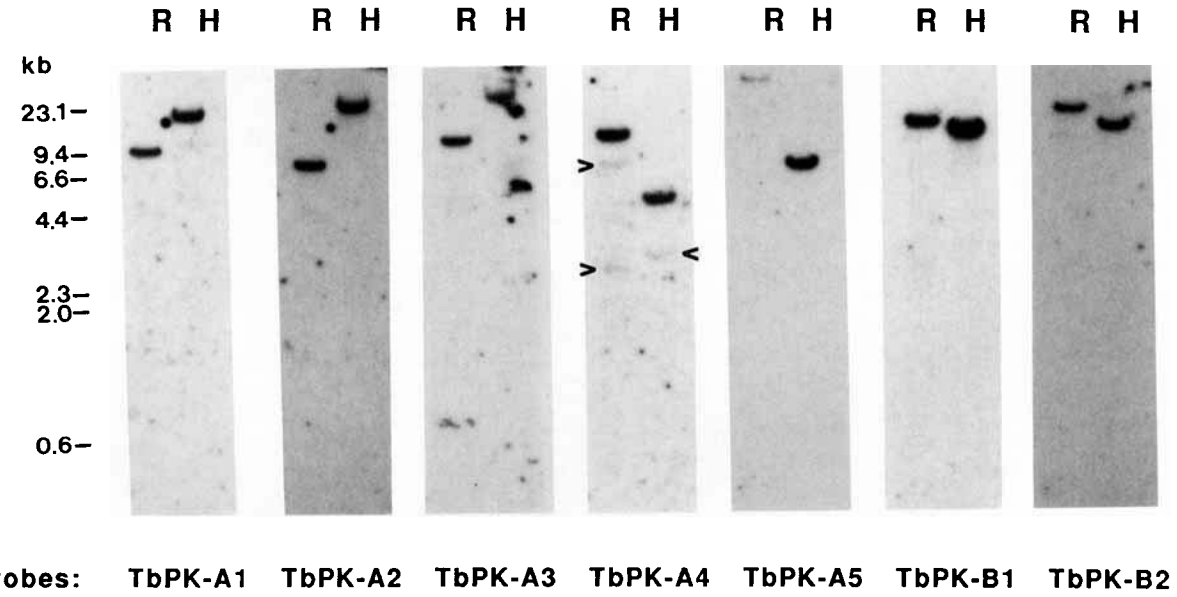

Fig. 2. Southern analysis of TbPK genes. Genomic DNA (3 μg) isolated from the procyclic form of *T. brucei* was digested with Hind III (H) or EcoR I (R), fractionated by gel electrophoresis, blotted onto nitrocellulose membrane, and probed individually with isolated cDNA inserts of TbPKs. DNA molecular size markers are indicated in kilobase pairs (kb) on the left. The calculated molecular sizes (in kb) of the hybridized restriction fragments are 8.3 (R) and 13 (H) for TbPK-A1; 7.2 (R) and 22 (H) for TbPK-A2; 10 (R) and 24 (H) for TbPK-A3; 10, 6.8, 2.6 (R), and 4.6, 2.9 (H) for TbPK-A4; 30 (R) and 6.2 (H) for TbPK-A5; 12 (R) and 10 (H) for TbPK-B1; and 13 (R) and 9.4 (H) for TbPK-B2, respectively. The weak signal of the fragment (30 kb) hybridized to TbPK-A5 may be due to the low transferring efficiency to the membrane from agarose gel because of its large size. The weak hybridization signals on the blot probed with TbPK-A4 (indicated by arrow heads) may represent other members of a subfamily in protein kinases family (see text).

the procyclic cellular DNA, indicating that they are all derived from *T. brucei*. Each TbPK probe demonstrated a unique hybridization pattern, and did not cross-hybridize with the other TbPK genes under high-stringency conditions. All TbPK probes, except TbPK-A4, hybridized to a single band of unique length on the DNA gel blots (Fig. 2). These six TbPK cDNA clones are thus likely to be each encoded by a single gene within the *T. brucei* genome. TbPK-A4, which

hybridized to one band with high intensity and 1–2 bands with lower intensities in the DNA lanes digested with Hind III and EcoR I, respectively, may be a member of a subfamily in the protein kinase family.

Differential Expression of TbPK Genes

To study the potential functions of these putative TbPKs, we first investigated the expression of these genes in different forms of the trypanosome. Poly(A)$^+$ RNA blots of bloodstream and procyclic forms of trypanosomes were probed individually with each of the seven TbPK cDNA inserts. To confirm the equal loading of RNA on the blots, the TbPK probes were washed-off, and the blots were re-probed for the abundant RNA of α- and β-tubulins [Sather and Agabian, 1985] as internal controls. The Northern hybridization profiles revealed that transcripts of TbPK-A2, TbPK-A5, and TbPK-B2 accumulated to similar levels in both bloodstream and procyclic forms. This is also true for the mRNAs detected by TbPK-A4 (4.2 kb on the RNA blot) and TbPK-B1 (~ 3.8 kb on the blot) (data not shown). However, the signals are weaker than those on the TbPK-A5 blot, suggesting that the TbPK-A4 and TbPK-B1 transcripts are less abundant in both forms of trypanosome. The mRNA of TbPK-A1, on the other hand, was approximately 3–4 fold more abundant in the bloodstream form than in the procyclic form (Fig. 3). In the TbPK-A3 hybridization profile, two RNA bands (2.2 kb and 2.5 kb, respectively) were observed in the bloodstream form of trypanosome, in addition to the band of 7.4 kb observed in both bloodstream and procyclic forms (Fig. 3). Thus, TbPK-A1 and TbPK-A3 demonstrated different accumulations of transcript in the two forms of *T. brucei*.

Analyses of *KFR1* Gene in *T. brucei*.

Using TbPK-A1 as a probe, we screened a cDNA library of *T. brucei* procyclic form. Out of nine positive clones obtained, the restriction maps of two clones containing the longest inserts (~ 1.3 kb) were determined. Nucleotide sequence analyses indicated that one clone is 14 bp longer than the other at the 5′ terminus, while the rest of the sequence is identical. The nucleotide and its deduced amino acid sequences of the longer clone are shown in Figure 4. It is 1241 bp in length and contains a short *trans-*

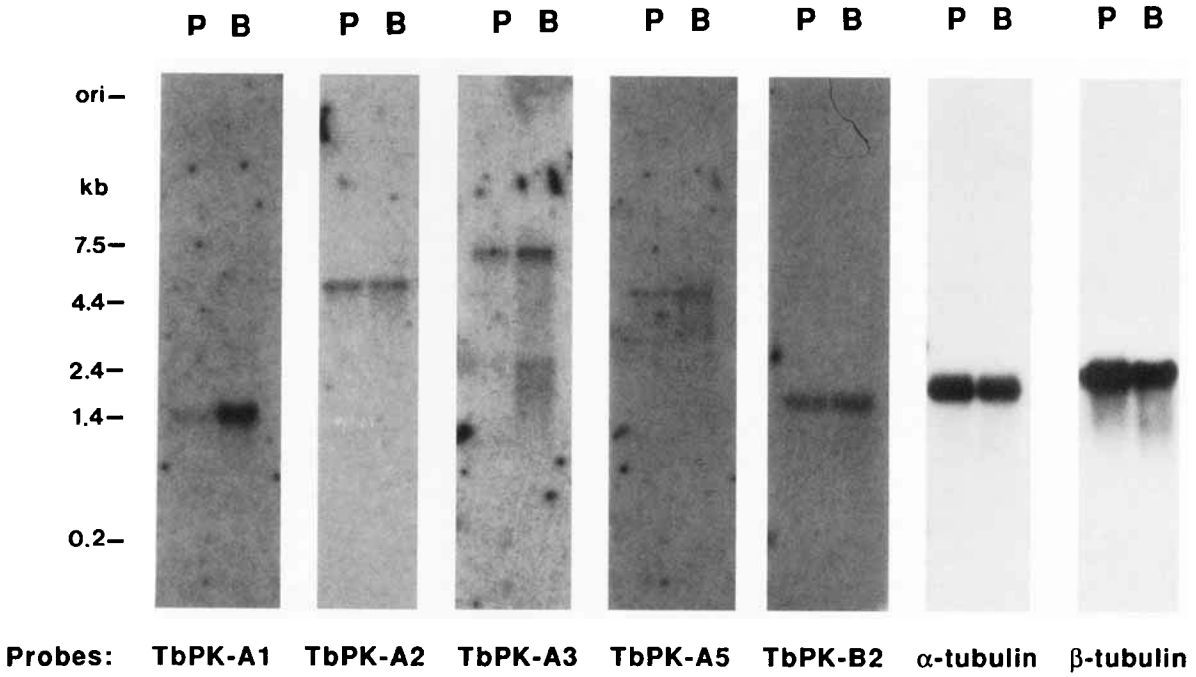

Fig. 3. Northern analysis of TbPK transcripts. Poly(A)$^+$ RNA (1 μg) isolated from procyclic (P) and bloodstream (B) form of *T. brucei* was separated on a denaturing agarose gel, blotted onto a nitrocellulose membrane and probed with TbPK inserts individually. RNA molecular size ladder (BRL) is indicated in kilobases (kb) on the left. The sizes of the hybridized transcripts are 1.4, 5.4, 5.2, and 1.7 kb for TbPK-A1, TbPK-A2, TbPK-A5, and TbPK-B2, respectively. TbPK-A3 hybridized to transcripts of 7.4, 2.5, and 2.2 kb, respectively. RNA blots hybridized with α- and β-tubulin probes are also included as controls.

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GTTTCTGTACTATATTGCAAAGTAGGAAAA CTAGATGGTGTCGTTCAGCATCGATGGACG AATTGAACAGCGGTACAAAATATTGTGCCA    90
                             M  V  S  F  S  I  D  G  R  I  E  Q  R  Y  K  I  L  C  H  (19)
                                                    TbKH2

CATTGGAAGCGGGGCGTACGGAGTCGTTTG GTGTGCTCTTGACAGGGTGACAAAAATGAA AGTGGCAATCAAAAAGGTATACGACGCATT   180
  I  G  S  G  A  Y  G  V  V  W  C  A  L  D  R  V  T  K  M  K  V  A  I  K  K  V  Y  D  A  F  (49)

TGGAAATAGACAAGACGCGCAACGAACCTA CCGCGAGGTGATGCTCCTTAGCACTCTGCA GCTCGACAACATTGTTCCTTTGCTAAATGT   270
  G  N  R  Q  D  A  Q  R  T  Y  R  E  V  M  L  L  S  T  L  Q  L  D  N  I  V  P  L  L  N  V  (79)

AATTCGCTCCATCAATGGAACGGACCTCTA TCTCGTATTTGAACTTGCCGAAACTGATTT GTCGGTTGTACTGCGGCATAACATCATGGA   360
  I  R  S  I  N  G  T  D  L  Y  L  V  F  E  L  A  E  T  D  L  S  V  V  L  R  H  N  I  M  E  (109)

GTCCGTACAGCGGCAATACGTAGCTTACCA AATTGTCTATGCTGTTGCCGGTTTGCACGC CCGTGGGGTGATCCATCGGGACTTGAAGCC   450
  S  V  Q  R  Q  Y  V  A  Y  Q  I  V  Y  A  V  A  G  L  H  A  R  G  V  I  H  R  D  L  K  P  (139)

TGCCAATGTTTTTCTGAACTCAGACTGCAA CATCAAGCTGGGCGACTTTGGCCTCGCGCG CTGCTTCAATACACAAGGTGGCGACAACGA   540
  A  N  V  F  L  N  S  D  C  N  I  K  L  G  D  F  G  L  A  R  C  F  N  T  Q  G  G  D  N  D  (169)

CCTCACTGAATACATAGCAACGCGCTGGTA TCGATCGCCAGAGGTTCTCGTGAAGTCCAC GTGCTACACAACAGCTATGGACATGTGGGC   630
  L  T  E  Y  I  A  T  R  W  Y  R  S  P  E  V  L  V  K  S  T  C  Y  T  T  A  M  D  M  W  A  (199)

CGTTGGTTGCATTTTGGGTGAATTGTTTAC CGGTTCCCCTTTGTTCACGGGGAACTCAAC ATTGCACCAAATCGGGTTGATTGTTGCAGC   720
  V  G  C  I  L  G  E  L  F  T  G  S  P  L  F  T  G  N  S  T  L  H  Q  I  G  L  I  V  A  A  (229)

CCTTGGCGAGCCATCGGCCGAGGACCTCGA GAGTTTGAAATCCGAAGAAACTTGGCCACT CATAGATTCTCTGCCTGCGATTGACCCAGA   810
  L  G  E  P  S  A  E  D  L  E  S  L  K  S  E  E  T  W  P  L  I  D  S  L  P  A  I  D  P  D  (259)

TCCGTTACCTGAGCGGCTGAGTAAATACAA CGCAGACGCCGTGGACTTAATATGTAAATG TATTGTCTTTGATCCCAACAAGCGGCCAAC   900
  P  L  P  E  R  L  S  K  Y  N  A  D  A  V  D  L  I  C  K  C  I  V  F  D  P  N  K  R  P  T  (289)

GGCGCGGGAGGCTCTGCAGCACCCTTACTT TGCTCCTTTTACGTCCCCGAGTGACTTCGA GGAGCTCGACAAGGCTGTGCCCATCAAACT   990
  A  R  E  A  L  Q  H  P  Y  F  A  P  F  T  S  P  S  D  F  E  E  L  D  K  A  V  P  I  K  L  (319)
                                  TbKH3

CCCATTTCCGGATGAGGAGGAGAGACCCGC GGATCAATATAGGAGTGCGTTATACGATAT TATTGGAAACCGTCGAAAGCAAAAGTTAGC  1080
  P  F  P  D  E  E  E  R  P  A  D  Q  Y  R  S  A  L  Y  D  I  I  G  N  R  R  K  Q  K  L  A  (349)
                                                    TbKH1

CTTGTAAACAGGACTGTCCAGCGGGCGAGC GCTTGTGGTGGGTCTGTGTTTTTGATGGCC CCCATCACGTCGCTCACGAAATGTTGCTCA  1170
  L  *                                                                                          (350)

GCCATATGGGACCTCCCACCCACATGCACA TATACATCATATACACCGTATCTTGGGTAG GTCAGAAAAAA                      1241
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Fig. 4. Nucleotide sequence and predicted amino acid sequence (single-letter code) of *KFR1* cDNA. DNA sequence of *trans*-splicing leader sequence is underlined. Amino acid sequence corresponding to that of the PCR clone, TbPK-A1, is also underlined. The peptide sequences for generating antisera are double-underlined.

spliced leader sequence, which indicates that the clone is a full-length cDNA [Sather and Agabian, 1985]. It encodes a putative serine/threonine protein kinase containing 350 amino acid residues with a predicted molecular mass of 40 kDa. All eleven conserved regions of protein kinases are present in this sequence. The sequence of TbPK-A1 (the probe for the library screening) is also observed with a total of four mismatches at the amino acid level in the primer regions, whereas the DNA sequence between the primers is identical to the corresponding fragment within the full-length cDNA clone (Fig. 4), indicating that the full-length cDNA clone and TbPK-A1 are derived from the same gene. A database search revealed that this protein has high sequence similarity to *KSS1* [Courchesne et al., 1989] and *FUS3* [Elion et al., 1990] protein kinases from *S. cerevisiae* (42% and 40% identity, respectively, excluding the carboxy-terminal 40-amino-acid residues), as well as to rat MAP kinase or ERK1 (37% identity) [Boulton et al. 1990] (Fig. 5). We therefore designated the gene of this protein *KFR1* (*KSS1* and *FUS3* related protein kinase 1). The homology is confined to the first 310 amino acids. Amino acid sequences at the carboxy-termini among these four protein kinases are quite divergent (Fig. 5). The carboxy-terminal 40-amino acid residues of *KFR1* do not have any significant sequence simi-

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KFR1  MVSFSIDGRIEQRYKILCHIGSGAYGVVWCALDRVTKMKVAIKKVYDAFGNRQDAQRTYREVMLLSTLQ-LDNIV  74
KSS1   MART·TFD·PSQ··LVDL··E····T·CS·IHKPSGI·····I-QP·SKKLFVT··I··IK··RYFHEHE··I  73
FUS3   MPKR·VYN·SSDFQLKSLL·E·····CS·THKP·GEI·····I-EP·DKPLF·L··L··IKI·KHFK-HE··I  72
MAPK  EVVKGQPFDVGP·YTQ·QY··E····M·SS·YDHVR·TR·····I-SP·EHQTYCQ··L··IQI·LGFR-HE·VI  89
                              I                    II                  III          IV

KFR1  PLLNVIRSINGTDL---YLVFELAETDLSVVLRH-----NIMESVQRQYVAYQIVYAVAGLHARGVIHRDLKPAN 141
KSS1  SI·DKV·PVSIDK·NAV···E··M····QK·INNQNSGFSTLSDDHV··FT···LR·LKSI·SAQ·····I··S· 148
FUS3  TIFNIQ·PDSFENFNEV·IIQ··MQ···HR·ISTQ-----MLSDDHI··FI··TLR··KV··GSN·····S· 142
MAPK  GIRDIL·APTLEAMRDV·I·QD·ME···YKL·KSQ-----QLSNDHIC·FL···LRGLKYI·SANVL·····S· 159
                              V                                                  VI
                                                  * *
KFR1  VFLNSDCNIKLGDFGLARCFNTQGGDND--------LTEYIATRWYRSPEVLVKSTCYTTAMDMWAVGCILGELF 208
KSS1  LL···N·DL·VC·····LASSSDSRETLV---GFM···V·····A··IMLTFQE·····I·SC····A·MV 220
FUS3  LLI··N·DL·VC·····IIDESAA··SEPTGQQSGM···V·····A···MLTSAK·SR···V·SC····A··F 217
MAPK  LLI·TT·DL·IC·····IADPEHDHT-------GF····V·····A··IMLNSKG··KSI·I·SV····A·ML 227
                VII                                  VIII                IX

KFR1  TGSPLFTGNSTLHQIGLIVAALGEP-SAEDLESLKSEETWPLIDSLPAIDPDPLPERLSK--YNADAVDLICKCI 280
KSS1  S·K···P·RDYH··LW··LEV··T·--F··FNQI··KRAKEY·AN··MRP·L·WETVWSKTDL·P·MI··LD·ML 294
FUS3  LRR·I·P·RDYR··LL··FGII·T·H·DN··RCIE·PRAREY·K···MYPAA··EKMFPR--V·PKGI··LQRML 290
MAPK  SNR·I·P·KHYLD·LNH·LGI··S·--Q···NCIINMKARNYLQ···SKTKVAWAKLFP·--SDSKAL··LDRML 299
                              X                                                  XI

KFR1  VFDPNKRPTAREALQHPYFAPFTSPSDFEELDKAVPIKLPFPDEEERPADQYRSALYDIIGNRRKQKLAL       350
KSS1  Q·N·D··IS·A···R···L·MYHD····P·YPPLNLDDEFWKLDNKIMRPEEEEEVPIEMLKDMLYDELMKTME 368
FUS3  V·D·A··I··K···E···LQTYHD·N··P·GEPIP·SFFEFDHHK·ALTTKDLKK·IWNEIFS             353
MAPK  T·N·N··I·VE···A···LEQYYD·T··PVAEEPFTFDMELD·LPKERLKELIFQETARFQPGAPEAP        367
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Fig. 5. Alignments of KFR1 sequence with products of *KSS1* and *FUS3* from yeast and MAP kinase from rat. Dashes indicate gaps introduced to optimize the alignment. Dots represent identities to the KFR1 sequence. Roman numerals indicate the conserved regions of the catalytic domain of protein kinases [Hanks et al., 1988]. The regulatory phosphorylation residues (threonine and tyrosine) of MAP kinase and the *KSS1* and *FUS3* proteins are indicated by asterisks.

larities to any of the proteins in the databases. The regulatory phosphorylation sites detected in the murine MAP kinase [Payne et al., 1991] and the *KSS1* and *FUS3* protein kinases [Gartner et al., 1992] are also present in the deduced sequence of *KFR1* (threonine-171 and tyrosine-173) (Fig. 5).

Our Southern hybridization results (not shown) confirmed that *KFR1* is a single gene in the genome of *T. brucei*. The genomic DNA samples of the trypanosome were digested with BamH I, Cla I, Hind III, Pst I, and Xho I, respectively, as well as the combinations of BamH I/Pst I, Hind III/Pst I, and Hind III/Pst I/EcoR I. There is a Xho I recognition site (position 746) in the cDNA sequence of *KFR1*. Southern hybridization of partially digested genomic DNA with Xho I, however, did not reveal any ladder pattern (not shown), suggesting that *KFR1* protein is not encoded by tandemly repeated genes. Thus, all the evidence suggests the presence of only a single copy of *KFR1* gene in the *T. brucei* genome.

An RNA blot probed with *KFR1* cDNA (not shown) demonstrated the same pattern as that probed with TbPK-A1, i.e., a single band of ~1.4 kb with a 3–4 fold higher level in the bloodstream than in the procyclic forms (Fig. 3). To facilitate the quantitation of the RNA, we carried out slot blot hybridization experiment with yeast tRNA as control (Fig. 6). The slot blot results confirmed that the mRNA of *KFR1* is 3–4 fold more abundant in the bloodstream form than in the procyclic form.

Differential Expression of KFR1 Protein

We generated antisera against synthetic peptides corresponding to carboxy-, amino-terminal, and internal regions of *KFR1* protein, named TbKH1, TbKH2, and TbKH3, respectively (see Materials and Methods and Fig. 4). When the anti-TbKH1 serum was used to probe the lysate of *E. coli* cells transformed with *KFR1* gene, a very strong signal at 40 kDa was observed. This signal, however, was absent in the cell lysate of nontransformed *E. coli* (Hua and Wang, unpub-

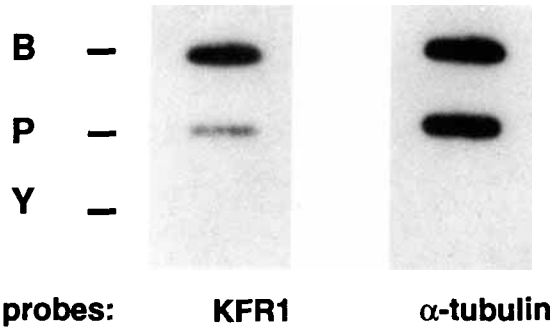

Fig. 6. Slot blot analysis of *KFR1* mRNA. Poly(A)$^+$ RNA (0.75 μg) isolated from bloodstream (B) and procyclic (P) form of *T. brucei*, and yeast tRNA (5 μg) (Y) were denatured and slot-blotted onto a nitrocellulose membrane. The blot was probed with *KFR1* cDNA (left). After washing-off the *KFR1* probe, the same blot was re-probed with α-tubulin DNA (right) to confirm equal loading.

lished data). The size of the protein band is same as the predicted molecular mass of KFR1 protein deduced from its amino acid sequence. This signal was not observed when the lysates of both transformed and nontransformed *E. coli* were probed with preimmune serum (data not shown). The TbKH1 sequence is located at the unique C-terminal region of KFR1 (Figs. 4, 5). These results strongly indicate that anti-TbKH1 serum specifically recognizes KFR1 protein. On the other hand, there were no significant signals detected when the antisera against TbKH2 and TbKH3 were used to probe with both transformed and untransformed *E. coli* cell lysates, nor with the *T. brucei* cell lysates (data not shown). We therefore used anti-TbKH1 serum in the following experiments.

Using anti-TbKH1 serum as a probe, we carried out Western blottings with serial dilutions (5–15 μg protein each lane) of total lysates of *T. brucei* procyclic and bloodstream forms (Fig. 7A). The presence of the 40 kDa *KFR1* protein was positively identified in both cell lysates. We also included Western blot probed with anti-α-tubulin antibody as an internal control (Fig. 7B). All the immunostaining signals demonstrated a linear relationship within the range of sample loadings. As shown in Figure 7B, 2 μl of the procyclic cell lysate and 4 μl of the bloodstream cell lysate revealed signals of equal intensity on the blot probed with the antibody against α-tubulin. Whereas the same ratio of lysates on the blot probed with anti-TbKH1 serum (Fig. 7A) demonstrated that the KFR1 protein is present at a 2-fold higher level in the bloodstream form than in the procyclic form. For example,

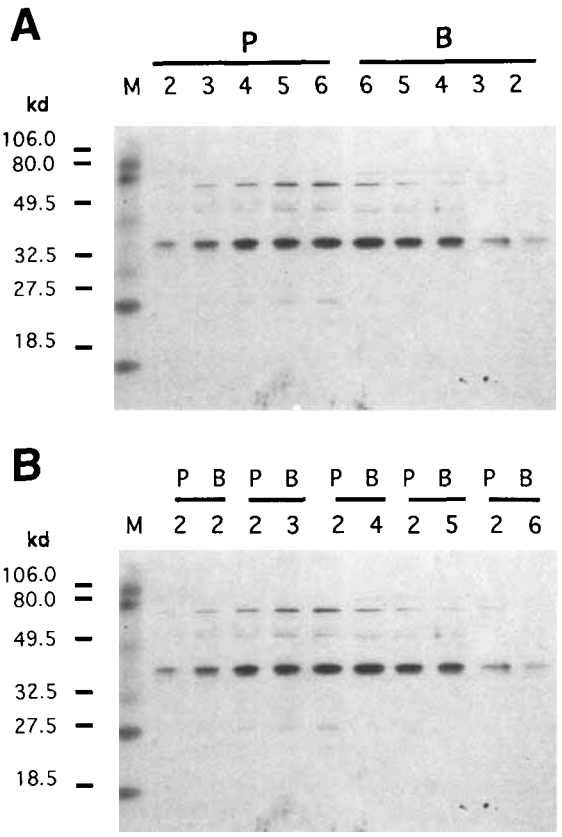

Fig. 7. Protein analysis. **A:** Total cell lysates isolated from procyclic (P) and bloodstream (B) form of *T. brucei* (2.5 mg/ml each) were fractionated on a 12.5% SDS-PAGE with a serial loading (as indicated in μl). After electroblotting onto a membrane, the lysates were probed with rabbit anti-TbKH1 peptide serum. The signals above 50 kDa and near 28 kDa are also observed on the blot probed with pre-immune serum. **B:** Fifty-fold diluted total cell lysates of (A) (loaded as indicated in μl) were immunostained with mouse monoclonal antibody against chicken α-tubulin.

the absorbance readings from laser densitometer scanning were 72.5 ± 1.1 (4 μl bloodstream form) and 32.8 ± 0.6 (2 μl procyclic form) (mean ± standard error). These results were reproduced at least four times. Thus, the KFR1 mRNA as well as its protein existed at higher levels in bloodstream form than in the procyclic form of *T. brucei*.

DISCUSSION

Using the PCR technique, we have identified seven distinct cDNAs encoding seven putative protein kinases from *T. brucei*. Our sequence analyses together with the Southern hybridization results indicate that there are at least seven protein kinase genes in the genome of *T. brucei*. This is consistent with the current view that

protein kinase genes in mammals, *Drosophila,* and yeast are complex families consisting of multiple genes of like classes of enzymes [Hunter, 1987]. A different set of primers and/or different reaction conditions may reveal even more protein kinase sequences in *T. brucei.*

It is interesting to notice that TbPK-B2 contains a very hydrophilic fragment (18 charged amino acids out of the total of 26 amino acids) between regions VII and VIII (Fig. 1B). The amino terminal half of this fragment is negatively charged, while the carboxy terminal half is mainly positively charged. There is an aspartic acid every 6–7 amino acids in this stretch. Secondary structure analysis [Chou and Fasman, 1978] suggests that this fragment may be a helix. Surface probability analysis [Janin et al., 1978; Emini et al., 1985] suggests that this fragment may stretch out on the surface of the protein. Interestingly, TbPK-B2 also contains a sequence that matches the bipartite nuclear targeting signal (Fig. 1B), i.e., two basic residues, a spacer region of 9–37 amino acids, followed by a second basic cluster in which at least three out of the next five amino acids are basic [Dingwall and Laskey, 1991]. There is much evidence suggesting that protein kinases are present in the nuclei of animals [Masmoudi et al., 1989], plants [Datta et al., 1985], and yeast [Toda et al., 1991]. For example, SPK1, a nuclear protein kinase of *Schizosaccharomyces pombe,* interacts with an AP-1-like transcription factor, and confers resistance to staurosporine [Toda et al., 1991], an inhibitor of protein kinase C (PKC) [Tamaoki et al., 1986], and other protein kinases [Nakano et al., 1987]. Indeed, phosphorylation has been widely implicated as a mechanism for modulating the activity of transcription factors that in turn regulate gene expression [Jackson et al., 1990]. One could thus speculate that TbPK-B2 may exist in the trypanosomal nucleus.

The amino acid sequence of *KFR1* shows high sequence similarity to *KSS1* and *FUS3* protein kinases (42% and 40% identity, respectively) of *S. cerevisiae* (Fig. 5). *KFR1* protein is also similar (37% identity) to rat MAP kinase or ERK1 [Boulton et al., 1990] and other related protein kinases. The characteristic motif "TEY" upstream of region VIII of MAP kinases [Nishida and Gotoh, 1993] also exists in the *KFR1* sequence (Fig. 5). Therefore, *KFR1* protein may represent the MAP kinase homolog in *T. brucei.* The growth-regulated properties of MAP kinase have been well documented; mitogens and various growth factors rapidly activate the MAP kinase and further promote the cell growth [see reviews by Nishida and Gotoh, 1993; Pelech and Sanghera, 1992; and references therein]. High-dose *KSS1* promotes recovery of *S. cerevisiae* from pheromone-induced growth arrest in G_1 phase [Courchesne et al., 1989]; while *FUS3* mediates cell cycle arrest during mating of the yeast [Elion et al., 1990]. Both *KSS1* and *FUS3* are regarded as MAP kinase homologs in yeast [Nishida and Gotoh, 1993; Thomas, 1992]. Since all the homologous proteins of *KFR1* in the databases are involved in cell growth control, one may speculate that *KFR1* also plays a role(s) in the cell growth of trypanosome. Our Northern and slot blot analyses (Figs. 3, 6) demonstrated that mRNAs of *KFR1* (TbPK-A1) accumulate differentially in the two different forms of trypanosome. The mRNA of *KFR1* is more abundant in the bloodstream form (3–4 fold higher) than in the procyclic form. On the other hand, Western blots indicated that the *KFR1* protein is present approximately 2-fold higher in the bloodstream form than in the procyclic form. The average generation time of the bloodstream form (6–8 h) of the trypanosome is considerably shorter than that of the procyclic form (approximately 20 h) (our observation). These notions suggest that increasing the activity of *KFR1* may contribute to an increase in the rate of cell divisions of trypanosomes.

MAP kinases are activated upon the phosphorylation of both threonine and tyrosine residues of the "TEY" motif. This activation is triggered by upstream regulators such as growth factor receptors with tyrosine kinase activity [Nishida and Gotoh, 1993; Pelech and Sanghera, 1992]. Tyrosine kinase activity [Parsons et al., 1991] and an epidermal growth factor receptor homolog [Hide et al., 1989] have been detected in the bloodstream form of *T. brucei.* Since the trypanosome is exposed to the growth factors in the host blood, there is a possibility that *KFR1* protein is more active in host blood than in tsetse fly due to the phosphorylation at the "TEY" motif through the growth factor signal cascade. Therefore, it would be interesting to determine whether the threonine and tyrosine residues of the "TEY" motif of the *KFR1* protein are also the regulatory phosphorylation sites.

The other putative protein kinase, TbPK-A3, that is differentially expressed in the two forms of *T. brucei* revealed additional, possibly processed, forms of RNA in the bloodstream form

(Fig. 3). TbPK-A3 has sequence similarity to the product of *SNF1* of yeast [Celenza and Carlson, 1986] and its related proteins from *Arabidopsis thaliana* [Le Guen et al., 1992] as well as to the myosin-light-chain kinase (MLCK) of *Dictyostelium* [Tan and Spudich, 1991] (Table 1). MLCKs phosphorylate the regulatory myosin light chain and thereby regulate the force-producing interaction of myosin with actin [Tan and Spudich, 1991]. *SNF1* plays a central role in carbon catabolite repression in yeast [Celenza and Carlson, 1986], and is required for the expression of several glucose-repressible genes in response to glucose deprivation. The *snf1* mutants are unable to utilize sucrose, galactose, or nonfermentable carbon sources [Celenza and Carlson, 1986]. The homology of the full-length TbPK-A3 to *SNF1* or MLCK as well as similarities in their biological function(s) are yet to be determined.

In summary, we have obtained seven distinct partial cDNA clones containing the conserved protein kinase sequences. Southern and sequence analyses of these clones indicated the presence of multiple genes of protein kinases in *T. brucei*. More interestingly, two of the clones demonstrated differential accumulation patterns of transcripts at different stages of development of the trypanosome. The complete sequence of one of the cDNA clones *KFR1* showed high sequence identity to the yeast *KSS1* and *FUS3* protein kinases, and mammalian MAP kinase. It is postulated to play a role in accelerating the proliferation of *T. brucei* in the mammalian blood.

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