Supplemental data

Trypanosomes Have Six Mitochondrial DNA Helicases, with One Controlling Kinetoplast Maxicircle Replication

Beiyu Liu, Jianyang Wang, Nurit Yaffe, Megan Lindsay, Zhixing Zhao, Aviad Zick, Joseph Shlomai and Paul T. Englund

Experimental Procedures

Gene knock-out. ~500 bp each of the 5' and 3' untranslated regions from the PIF genomic sequences were PCR amplified and inserted into pKO (Lamb et al., 2001) (a gift from Dr. Jay Bangs, University of Wisconsin) containing resistance markers for blasticidin (BSR) or puromycin (PUR). The two alleles were replaced with pKO_{PUR} and pKO_{BSR} sequentially in 29-13 cells that constitutively express the T7 RNA polymerase and the tetracycline repressor. In case the gene product was essential for trypanosome growth, an ectopic copy of the gene was introduced into the cells before replacement of the second allele. To introduce the ectopic copy, the entire gene including the stop codon was inserted into the pLew79-MHTAP vector (Jensen et al., 2007) which expressed the gene product downstream of a T7 promoter under the control of a tetracycline operator. After transfection into the pKO_{PUR} trypanosomes, ectopic protein expression was maintained by including 1 μg/ml tetracycline in the culture medium. When overexpression was toxic for cell growth, reduced concentrations of tetracycline (2 ng/ml, 5 ng/ml, 10 ng/ml and 100 ng/ml) were used. pKO_{BSR} finally was introduced into the cell to knock out the second allele while cell viability was maintained by expression of

the ectopic copy. Expression of the ectopic copy can be turned off by removal of tetracycline from the culture.

Expression and purification of Recombinant TbPIF2. We prepared two constructs for expressing TbPIF2. One was a GST fusion and the other, a deletion, was truncated at its N-terminus. For both proteins we made a single mutation (K462A) in the Walker A motif, a sequence involved in ATP binding. This mutation was made by PCR using primers listed in Table S2.

For the GST-TbPIF2 and corresponding mutant, the coding sequences (minus the first 41 amino acids which constitute a predicted mitochondrial targeting signal) were amplified by PCR, cloned into GST fusion vector pGEX6P-2 (GE Healthcare), and transformed into the *E. coli* Rosetta^{TM} (DE3) pLysS strain (Novagen). The strain was inoculated into 1 L LB medium with 34 μg/ml chloramphenicol, 100 μg/ml ampicillin, and grown to an OD_{600nm} of 0.6. After addition of 0.1 mM IPTG, the culture was incubated for another 3 h at 25 °C. Cells were harvested by centrifugation (8000 g, 10 min) and the pellet was resuspended in 10 ml PBS. After lysis by sonication, the suspension was centrifuged (10000 g, 30 min) and the supernatant was mixed with 0.4 ml Glutathione Sepharose 4B (GE Healthcare) (1 h, 4 °C). The beads were then washed four times with 1 ml PBS. Proteins were eluted 3 times with 0.3 ml buffer A (50 mM Tris-HCl, pH 8.0, 10 mM reduced glutathione). The eluates were loaded onto a 0.5 ml column of heparin-Sepharose FF (GE Healthcare) equilibrated with buffer B (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM DTT). Proteins were eluted with 0.8 M NaCl and dialyzed against buffer C (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT).

For the truncated TbPIF2 and its K426A mutant, we used an approach previously applied to the yeast PIF helicase, Rrm3p (Ivessa et al., 2002). Coding sequences (minus the first 416 amino acids), were amplified by PCR, cloned into pET28a (Novagen), and transformed into the *E. coli* Rosetta^{TM} (DE3) pLysS strain (Novagen). The strain was inoculated into 500 ml LB medium with 30 μg/ml kanamycin, and grown to an OD_{600nm} of 0.7. After addition of 0.1 mM IPTG, the culture was incubated for another 4 h at 17 °C. Cells were harvested by centrifugation (8000 g, 10 min) and the pellet was resuspended in 5 ml buffer A (50 mM sodium phosphate, pH 8.0, 800 mM NaCl, 10 mM imidazole). After lysis by sonication, the suspension was centrifuged (10000 g, 30 min) and the supernatant was mixed gently with 0.5 ml Ni-NTA slurry (Qiagen) (1 h, 4 °C). The Ni-NTA beads were then washed with 5 ml buffer B (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole, 20 mM β-mercaptoethanol). Proteins were eluted 4 times with 1.5 ml buffer C (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 250 mM imidazole, 20 mM β-mercaptoethanol). The eluates were diluted 1:3 with buffer D (25 mM sodium phosphate, pH 7.5, 300 mM NaCl, 1 mM DTT). The samples were loaded onto a 0.25 ml Fractogel EMD heparin 650(s) column (Merck) equilibrated with the same buffer. The column was washed with 3 bed volumes of buffer D and eluted with 2 bed volumes of buffer D containing 800 mM NaCl. The eluted fraction was desalted on a Zeba Desalting Spin column (Pierce).

Supplementary References

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Table S1. Localization of TbPIF1-8 and effects of RNAi, knockout, and overexpression

Protein	Genebank accession no.	Predicted protein sizes (amino acids)	Protein localization	RNAi[1]	Knock-out[2]	Overexpression[3]
TbPIF1[4]	XP_828762	929	Mitochondrion	Cell death, kDNA loss[a]	Not done	No effect
TbPIF2[4]	XP_828763	1048	Mitochondrion	Cell death, kDNA defect [b]	Single allele, no effect	Growth defect, kDNA defect
TbPIF3	XP_829242	878	Cytoplasm	No effect[a]	Double alleles, no effect	No effect
TbPIF4	XP_829537	819	Mitochondrion (kDNA network)	No effect[a,b]	Failed	No effect
TbPIF5	XP_847187	928	Mitochondrion (antipodal sites)	No effect [a,b]	Single allele, no effect	Growth defect, kDNA loss
TbPIF6	XP_822349	818	Nucleus	No effect[a]	Single allele, no effect	No effect
TbPIF7	XP_846907	992	Mitochondrion (kDNA network)	No effect[a]	Double alleles, no effect	Growth effect
TbPIF8	XP_845724	694	Mitochondrion (kDNA network)	Cell death [b]	Single allele, no effect	No effect

[1]RNAi of PIFs. We used two different RNAi vectors, pZJM (superscript a) and the stem-loop vector (superscript b) (Wang et al., 2000), to produce dsRNA upon induction with tetracycline. The % depletion of mRNA caused by RNAi were: TbPIF1, 70% (pZJM); TbPIF2, 90% (stem loop); TbPIF3, 5% (pZJM); TbPIF4, 50% (pZJM), 80% (stem loop); TbPIF5 90% (pZJM), 90% (stem loop), and TbPIF8, 80% (stem loop). Only with TbPIF1, TbPIF2 or TbPIF8 did RNAi cause a growth defect.

[2]Knock-out of PIFs. We first attempted to replace the two alleles of each gene by homologous recombination with a puromycin or blasticidin resistance gene. This strategy was successful for TbPIF3 and TbPIF7 as judged by Southern blot and Northern blot (supplementary Fig. 3); neither knockout affected growth, indicating that these two genes are not essential for growth. Attempts to replace both alleles of other PIFs with two different drug markers failed (data not shown). For TbPIF2, 5, 6 and 8, only one allele could be replaced. Because knockout of both alleles may be lethal, we introduced an ectopic copy into the cell so this ectopic gene, which express PIF upon induction with tetracycline, should allow the replacement of the second allele (Jensen et al., 2007). This strategy was also unsuccessful using tetracycline concentration ranging from 2-100 ng/ml (1 μg/ ml tetracycline is toxic for trypanosome, see below). For TbPIF4, neither allele could be knocked out for unknown reasons.

[3]Overexpression of PIFs. Overexpression of TbPIF2, TbPIF5 or TbPIF7 upon induction with 1 μg/ ml tetracycline is toxic for trypanosomes. For these proteins, the fold increase in mRNA was ~10, ~15 and ~14 respectively. Remarkably, overexpression of TbPIF2 or TbPIF5 also causes shrinkage of kDNA network, indicating that too much protein may also reduce kDNA stability. We did not observe any kDNA defect by DAPI staining for

the cell line with TbPIF7 overexpression although the cell growth is also affected after induction. There is no growth effect following overexpression of the other 5 PIFs.

[4]TbPIF1 and TbPIF2 genes are tandemly linked on chromosome 11.

Table S2. Primers used in this paper.

Gene	Purpose	Primer sequence
TbPIF1	Localization	5'- CGCTAAGCTTATGTTGCGTCGGCTCCTC -3' 5'- CGCTTGGCTAGCTTCCAACGACTC -3'
	RNAi	pZJM vector 5'- CGCTAAGCTTATGTTGCGTCGGCTCCTC- 3' 5'- CGAACTCGAGGATGTCTCCGAT- 3'
TbPIF2	Localization	pXsGFPFUS 5'- CGCTCTAGAATGTCTTCAAAGACGGTAG -3' 5'- CGCTTCGCTAGCCGGTGTTGCTTC -3' *C-Myc* tagging 5'- GACCGGTACCGCACCCGAGGTAGATTTCGG -3' 5'- GCAGCTCGAGCGGTGTTGCTTCCTTCGC -3' 5'- GCAGGGATCCAATCATAGCCGGGTTGCCAC -3' 5'- GCATCATGGCGGCCGCAACACGAATTATTCCACCACTC -3'
	RNAi	Stem loop vector 5'- GCCGTTCTAGAATGTCTTCAAAGACGGTAGTTTGG -3' 5'- GCCGTAAGCTTGGTCCAGGCGGAAGCGCTACCGTAG -3' 5'- GCCGTACGCGTGGTCCAGGCGGAAGCGCTACCGTAG -3'
	Knock-out	5'- GCCGCTCGAGGAAGGGCACGAGTCAG -3' 5'- GCCGAAGCTTTGGTCCGACTGCACTATC -3' 5'- GCCGACTAGTAATCATAGCCGGGTTGCC -3' 5'- GCATGCAGGCGGCCGCAACACGAATTATTCCACC -3'
	Overexpression	5'- GCAGCCTAGGATGTCTTCAAAGACGGTAG -3' 5'- GCAGCTCGAGTTATTACGGTGTTGCTTCCTTCGC -3'
	Point mutation (K462A)	5'- GGGGCAGGGACTGGAGCGTCTCTTCTTCTGCGGG -3' 5'- CCCGCAGAAGAAGAGACGCTCCAGTCCCTGCCCC -3' 5'- GCAGCCTAGGATGTCTTCAAAGACGGTAG -3' 5'- GCAGCTCGAGTTATTACGGTGTTGCTTCCTTCGC -3'
	Expression & Purification	pGEX-6P-2 (GST fusion) 5'- GCAGGGATCCTCTACCCCGTTATCACTGATTGCGG-3' 5'- CAGACTCGAGTTATTACGGTGTTGCTTCCTTC -3' pET-28a (ΔNTbPIF2) 5'- TCAGGCTAGCGCTATAAACGGTGAAATGACATAC -3' 5'- CAGACTCGAGTTATTACGGTGTTGCTTCCTTC -3'
TbPIF3	Localization	5'- GCGGATATCATGCGCCTGGCTGCTGAATC -3' 5'- TGCTCTAGAGTCCGTCAAACCAAC -3'
	RNAi	pZJM vector 5'- GCGAAGCTTGACACCTTTGTCAATCC -3' 5'- AGTCTCGAGGACGCGACGCCC -3'
	Knock-out	5'- GCCGCTCGAGCGCCTTCCCGAACATGTCTGTGTAC -3' 5'- GCCTGAATTCGCAGCAGGTGGGAGATGAATGCAC -3' 5'- GCAGACTAGTTTACTAGGTATATTCTTCACTATCC -3' 5'- GCATGCAGGCGGCCGCTTGGTATGACGTGTGTTCGCATCC -3'
	Overexpression	5'- GCAGCCTAGGATGCGCCTGGCTGCTGAATCTTCTCTTG -3'

		5'- GCAGCTCGAGTCATCAGTCCGTCAAACCAACAAGTAAC -3'
TbPIF4	Localization	5'- TCGAAGCTTATGCTCCTCAACTCC -3' 5'- GCACGGCTAGCCTTGTGGTGTTTCC -3'
	RNAi	pZJM vector 5'- GCGAAGCTTGGGCGAGAAGGACG -3' 5'- GAGCTCGAGGATTCGTTTGTCTC -3' Stem loop vector 5'- GCCGTTCTAGAATGCTCCTCAACTCCACCAGGACTCTATTG -3' 5'- GCCGTAAGCTTGTGCTTCCCCCAATGTTGAGTGCAGC -3' 5'- GCCGTACGCGTGTGCTTCCCCCAATGTTGAGTGCAGC -3'
	Knock-out	5'- GCCGGGTACCGCATAGAACTTCGGAACTGC -3' 5'- GCCGAAGCTTGACGTGGCTCGAGCATTAAC -3' 5'- GCCGACTAGTGATATCGTTTTTCGTGCC -3' 5'- GCCGGCATGCGGCCGCGTACACCTTCTTCCGTTTC -3'
	Overexpression	5'- GCAGCCTAGGATGCTCCTCAACTCCACCAGGACTCTATTG -3' 5'- GCAGCTCGAGTTATCACTTGTGGTGTTTCCACGTCGC -3'
TbPIF5	Localization	5'- TCGAAGCTTATGCTCTCCCGTCTAAG -3' 5'- ACGCCTAGGTTCTTCCACTTCCCC -3'
	RNAi	pZJM vector 5'- GCGAAGCTTGTTGACTTCACCAC -3' 5'- GCGCTCGAGACTTCAGTAGGTTC -3' Stem loop vector 5'- GCCGTTCTAGAGTTGACTTCACCACATGCGGCAATCG -3' 5'- GCCGTAAGCTTACTTCAGTAGGTTCAGCGGCTG -3' 5'- GCCGTACGCGTACTTCAGTAGGTTCAGCGGCTG -3'
	Knock-out	5'- GCCGCTCGAGGAATGGGGAGCGCAG -3' 5'- GCAGAAGCTTTTGCGTGTTTATTTAAAAAC -3' 5'- GCCGACTAGTCCGAGAGCGATGAGCGAAAAAGACG -3' 5'- GCCTACTGGCGGCCGCCCATAAGCACCGTGGCACATTTATG -3'
	Overexpression	5'- GCAGCCTAGGATGCTCTCCCGTCTAAGCGCC -3' 5'- GCAGCTCGAGTTATTATTCTTCCACTTCCCCTTC -3'
TbPIF6	Localization	5'- CATGGTAGTACTGCGG -3' 5'- GGACGGCTAGCATCATCGTCCACC -3'
	RNAi	pZJM vector 5'- GCGAAGCTTGATATAGAAAAGAAC -3' 5'- GCACTCGAGGTAGAGGCGGTTAC -3'
	Knock-out	5'- GCCGCTCGAGTCGTCTGCGACGGGCTGCTGTGG -3' 5'- GCCGAAGCTTAATCCAATTTTCATGTAAAAC -3' 5'- GCCGACTAGTGGAAAGAAACTAAGAGATCACACAG -3' 5'- GCATGCAGGCGGCCGCACTTCCTCGCTGCTGTTGGTTCC -3'
	Overexpression	5'- GCAGCCTAGGATGGTAGTACTGCGGCCAAAG -3' 5'- GCAGCTCGAGTCATCAATCATCGTCCACCATTAG -3'
TbPIF7	Localization	5'- CTTGGATCCGATATCATGTGGGATGGGCCGCTACG -3' 5'- ACCACTAGTCGACCAGCAGCCGC -3' 5'- TCGACTAGTGGTAATTGAACCGCATCGATGGACAATG -3' 5'- ATAAGAATGCGGCCGCTCTAGAGTTCACCCTTCGGGCGCG -3'
	RNAi	pZJM vector 5'- GCGAAGCTTCGCTCGTCTGGTTG -3' 5'- AGCCTCGAGTCCTCTCTGTTTCGACGC -3'
	Knock-out	5'- GCCGGGTACCTTTACCCCATTGTTATCTTATTTTTG -3' 5'- GCCGAAGCTTCTGAGAAAAATTCCACAAAC -3' 5'- GCCGACTAGTCTCTATGCATGGCACTCAGGTGGG -3' 5'- GCATGCAGGCGGCCGCATGTAATGTTAGAAGAAGATAG -3'
	Overexpression	5'- GCAGCCTAGGATGTGGGATGGGCCGCTACGAAGTC -3'

		5'- GCAGGGATCCTCATCAGTTCACCCTTCGGGCGCG -3'
TbPIF8	Localization	5'- GACAAGCTTATGACACGTTGCCGGCTC -3' 5'- CCGCTCGAGTGGGATCCGCGTGTTGCATACGG -3' 5'- CCACTCGAGCGGCTTGGCCCCCACCAG -3' 5'- GCACCGCTAGCAGCTTCTCCACCGTTGC -3'
	RNAi	Stem loop vector 5'- TGCTCTAGAATGACACGTTGCCGGCTCGTTGGTG -3' 5'- TCGACGCGTTCCAACTGGTCTCGTGAGGGAATCT -3' 5'- TGCAAAGCTTTCCAACTGGTCTCGTGAGGGAATCT -3'
	Knock-out	5'- GCCGCTCGAGATGTGGTTATTTTAGATGATGAAGATATC -3' 5'- GCTGAAGCTTGTAACCTGTAGCGGCCCCCTATAAG -3' 5'- GCCGTCTAGAAGGAAGAGGCTACTAGTTTTTTTGGTG -3' 5'- GCATGCAGGCGGCCGCGGCATTTCCTATTTCAACCAAGAGGGG- 3'
	Overexpression	5'- GCAGCCTAGGATGACACGTTGCCGGCTCGTTGGTGCTTC -3' 5'- GCAGCTCGAGTCATCAAGCTTCTCCACCGTTGCACTG -3'

Legends

Figure S1. Multiple sequence alignments of TbPIF1-8. Sequences of TbPIF1-8, ScPif1 and ScRRM3 were aligned using the progressive method implemented in the software CLC Sequence Viewer 5 (downloaded from http://www.clcbio.com/index.php?id=28). Parameters used for these alignments were: gap open cost = 10; gap extension cost = 1; gaps at the ends of sequences are treated like those in internal sequences. Alignments were then annotated by GeneDoc (downloaded from http://www.nrbsc.org/). Only the region of the sequence containing conserved helicase domain is shown. Numbers at the beginning and the end of each sequence are the numbers of residues at each terminus of the non-conserved sequence; these sequences are not shown. TbPIF1-7 share a high degree of similarity in the seven helicase motifs (indicated by Roman numerals: I, Ia, II, III, IV, V and VI) (Hall and Matson, 1999) and three additional motifs (A, B and C) which are found in *Escherichia coli* helicase RecD (Boule and Zakian, 2006) but diverge greatly in other regions. TbPIF1-3 have one extra

amino acid in Motif V compared to the yeast PIFs. The smallest trypanosome PIF, TbPIF8, diverges markedly from other PIFs. Since the sequences of TbPIF1-8 to ScPIF1 and ScRRm3 are similar, we cannot tell which TbPIF more closely resembles the yeast proteins. Residues highlighted in red are 100% conserved, those in orange are 80% conserved, and those in light blue are 60% conserved. +, hydrophobic residue; o, hydrophilic; x, any residue. Like *T. brucei*, the *T. cruzi* genome predicts 8 homologs, whereas that from *Leishmania major*, a related parasite, predicts 7 (the TbPIF5 homolog is missing).

Figure S2. Localization of PIF1-8. Live cells stably expressing TbPIF1-8 with a C-terminal GFP tag were stained with DAPI and analyzed by fluorescence microscopy. GFP is in green, and DAPI in red. Bar, 5 μm.

Figure S3. Southern and Northern blot analyses of TbPIF3 and TbPIF7 knock-out cell lines. After digestion with indicated restriction enzymes, total cellular DNA (1×10^6 cell equivalents/lane) was fractionated on a 1% agarose gel and a Southern blot was probed for TbPIF3 (A) or TbPIF7 (B). Total RNA (2×10^7 cell equivalents /lane) was fractionated and a Northern blot was probed for TbPIF3 (A) or TbPIF7 mRNA (B). TbPIF1was used as a loading control for both Southern blot and Northern blot (Load).

Figure S4. Gapped maxicircles increase after TbPIF2 overexpression (OE). (A) Isolated kDNA networks were labeled using TdT and fluorescein-12-dUTP (which indicates the location of gapped circles) and stained with DAPI. Upper panel shows

fluorescence micrograph of wild type cells with characteristic polar TdT labeling (Guilbride and Englund, 1998). Networks not labeled with TdT are not undergoing replication (network a is an example). Because of increased TdT label, networks b, c, and d have undergone progressively greater extents of replication. Lower panel shows network from cells after 1 day of TbPIF2 overexpression. Network shape is sometimes altered, and DAPI staining is not uniform, probably due to condensation of the central region of the networks (See EMs in Fig. 4A, 4B). There are few networks with polar labeling characteristic of wild type. Bar, 8 μm. (B) The percentage of TdT-positive cells was quantitated by visual analysis of more than 150 randomly selected cells. Error bars represent ±SD from three independent experiments. A two-tailed t test indicates a significant difference ($p < 0.01$) between wild type (Wt) and overexpression (TbPIF2 OE). (C) Determination of whether the extra gaps after TbPIF2 overexpression are in minicircles or maxicircles. For fluorescence *in situ* hybridization, cells were fixed, probed for minicircles or maxicircles, and the minicircle and/or maxicircle positive cells were quantitated. Using these conditions only the gapped circles are detected. Upper panel shows a characteristic wild type cell with the gapped minicircles in the polar region and the maxicircles concentrated in the center (Li et al., 2008). For merged pictures, minicircle is in red, maxicircle in green and DAPI in blue. Inset. Enlargement of minicircle and maxicircle signals. Bar, 5 μm. Lower panel shows a cell in which TbPIF2 had been overexpressed for two days. There is strong maxicircle expression, but the minicircle fluorescence is non-specific and detectable only because of the long exposure used. (D) Quantitation of FISH results from analysis of 100 cells before (day 0) and after TbPIF2 overexpression (day 2). Error bars represent ±SD of three independent

quantitations; A two-tailed t test indicates a significant difference between day 0 and day 2 for percentage of cells with gapped maxicircles ($p < 0.01$) but not for cells with gapped minicircles ($p > 0.1$). These results indicate that gapped maxicircles but not gapped minicircles increase after TbPIF2 overexpression.

Figure S5. Helicase assay of recombinant TbPIF2. (A) Assessment of purity of GST-TbPIF2 and mutant GST-TbPIF2 (K462A) using a Coomassie blue-stained SDS-PAGE gel. (B) Helicase assays of GST-TbPIF2 and mutant GST-TbPIF2 (K462A). The substrate was a circular M13mp18 single-stranded DNA to which a 5'-^{32}P-labeled 36-mer oligonucleotide had been annealed as described (Zhou et al., 2002). Assays (20 µl each) contained 200 ng recombinant protein, 50 mM Tris-HCl, pH 8.5, 2 mM DTT, 2 mM $MgCl_2$, 2 mM ATP, 0.25 mg/ml bovine serum albumin, and the substrate (15 fmol). Reactions were incubated at 37 °C for 10 min and subjected to electrophoresis with a 12% polyacrylamide gel in 0.5 × TBE (150 V, 1h). (C) Assessment of the purities of the truncated enzyme, ΔNTbPIF2 (missing 416 amino acids from the N-terminus) and the corresponding mutant, ΔNTbPIF2 (K462A), by a silver-stained SDS-PAGE gel. (D) Helicase assays of ΔNTbPIF2 and ΔNTbPIF2 (K462A). Single-stranded circular M13mp8 DNA (7.5 pmol) was mixed with 5'-^{32}P-labeled 48-mer (5 pmol; 5' TTCGAACCCTGCAGGTCGACG GATCCCCGGGAATTCGTAATCATGGTC 3'), of which 40 nucleotides are complementary to the M13mp8 sequence at positions 6219-6258, while 8 nucleotides at its 5'-end are unpaired. The annealing reaction (10 µl; 20 mM Tris-HCl, pH 7.6, 20 mM $MgCl_2$, 10 mM NaCl) was heated at 95 °C for 10 min and cooled at the rate of 1 °C/min. Helicase assays were carried out in a 20 µl reaction mixture containing 50 mM Tris-HCl, pH 7.6, 10

mM DTT, 3 mM ATP, 0.5 mg/ml bovine serum albumin, the substrate (118 fmol) and 0.25 mM $MgCl_2$ (as indicated in Results, inclusion of 10 mM $MgCl_2$ enhanced an accompanying nucleolytic activity, which interfered with the assay). Reactions were started by addition of the enzyme fraction, incubated at 30 °C for 30 min, and the products resolved by electrophoresis on a 8% polyacrylamide gel, in 0.5 × TBE for 3 h at 250 V at 0 - 2 °C. The lanes on each panel were run on the same gel but some irrelevant lanes were removed. The gels in panels B and D were analyzed by autoradiography.