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The *Trypanosoma brucei* DNA polymerase α core subunit gene is developmentally regulated and linked to a constitutively expressed open reading frame

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ABSTRACT

As an initial step towards the characterization of replicative DNA polymerases of trypanosomes, we have cloned, sequenced and examined the expression of the *Trypanosoma (Trypanozoon) brucei brucei* gene that encodes the DNA polymerase α catalytic core (polα). The protein sequence contains the six conserved regions that have been recognized previously in eukaryotic and viral replicative DNA polymerases. In addition, we have identified a seventh region which appears to be conserved primarily in α-type DNA polymerases. The *T.brucei* DNA pol α core N-terminus is 123 and 129 amino acids smaller than that of the human and yeast homologue, respectively. The gene is separated by 386 bp from an upstream open reading frame (ORF) of 442 codons. Stable transcripts of the upstream sequence are detected in both dividing and non-dividing forms, while polα transcripts are detected principally in dividing forms. Allelic copies of the *T.brucei* polα region exhibit restriction site polymorphisms; one such sequence polymorphism affects the amino acid sequence of the *T.brucei* DNA polα core. The *T.brucei* polα region cross-hybridizes weakly with that of *T.(Nannomonas) congolense* and *T.(Duttonella) vivax*.

INTRODUCTION

African trypanosomes are flagellate parasitic protozoa which are of considerable medical and economic importance due to their ability to infect man and his domestic animals. These parasites undergo antigenic variation as a means of evading the immune system of their host (for recent reviews see 1–3). The spread of most species depends on bloodmeals taken by the tsetse fly (*Glossina* ssp.). Different developmental stages occur in the bloodstream and the insect. In *T.b.brucei* these include rapidly dividing, long slender bloodstream forms which, through intermediate forms, differentiate to non-dividing stumpy bloodstream forms (4, 5). The latter, after entering the tsetse fly midgut following a bloodmeal, transform to procyclic forms which actively proliferate. Since DNA synthesis is a potential target for the development of anti-trypanosomal drugs, a study of the replicative DNA polymerases of trypanosomes was warranted.

In eukaryotes investigated to date, similar DNA polymerases are involved in genome replication (for reviews see 6–9). Recent models of the replication fork are based upon studies of *in vitro* SV40 DNA replication (reviewed in 10–12). Two distinct DNA polymerases, α and δ, are implicated in lagging and leading DNA strand synthesis, respectively, and these enzyme subunits may interact at the replication fork to form a higher order complex (10, 12, 13). A revised nomenclature for eukaryotic DNA polymerases has recently been proposed and will be used throughout this paper (14).

The DNA polymerase α core, with a mol.wt. of approximately 180,000 Da in man and yeast, is complexed with two different primase subunits of approximately 50,000 Da and 60,000 Da (9), and is thought to synthesize the lagging strand. The function of a fourth subunit of 70,000–80,000 Da is unknown.

The leading strand is synthesized by the DNA polymerase δ core which has a mol.wt. of 125,000 Da in calf thymus and yeast (11, 15, 16) and is found complexed with a polypeptide of 48,000–55,000 Da. DNA polymerase δ is processive only when it is associated with an auxiliary protein of 36,000 Da, the proliferating cell nuclear antigen (17–19), and intrinsically possesses a 3′ to 5′ exonuclease activity that facilitates proofreading of newly synthesized DNA (20).

Recently the genes for the catalytic core enzymes of human and yeast DNA polymerases α and yeast DNA polymerase δ have

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been sequenced (21–23) and comparisons show that 6 regions are highly conserved. These regions are, to a variable extent, also conserved among viral DNA polymerases, most notably those from herpes simplex virus (HSV), Epstein-Barr virus (EBV), and cytomegalovirus (CMV; 21, 23–25). The overall sequence similarity between the human and yeast DNA polymerases α is more pronounced than that found between yeast DNA polymerases α and δ.

Based upon one of the conserved regions which occur between the human and yeast DNA polymerase α genes we have synthesized a single oligonucleotide to identify the *T.brucei* gene (polα). Sequence analysis of clones to which the oligonucleotide hybridized indicate that the encoded sequence indeed corresponds to an α-type DNA polymerase. The gene is closely linked at its 5′-end to an open reading frame (ORF) in the same orientation which, unlike polα, is constitutively expressed both in dividing and non-dividing forms of *T.brucei*.

MATERIALS AND METHODS

Trypanosomes

The genomic DNA clones described in this paper are derived from *Trypanosoma brucei brucei* MItat1.1C, a clone from the *T.b.brucei* 427 isolate (26). For Southern and Northern blot analysis DNA and RNA were isolated from *T.b.brucei* ILTat1.1, which is derived from stock EATRO795 (27); *T.b.rhodesiense* ETat1.8, from TREU164 (28); *T.congolense* ILNat2.1 (X4), a clone of C49 (29) and *T.vivax* IL2337, from KETRI2430 (30).

The different forms of bloodstream trypansosomes (4) were isolated by a method modified from Shapiro et al. (31) and Shapiro and Kimmel (32). Irradiated Spraque-Dawley rats (600 rad) were infected with 10^4 trypanosomes and the parasitaemia was monitored by microscopy of wet bloodfilms. The forms are recognized by length and the way the flagellum is situated. The flagellum is partially free of the undulating membrane in long slender forms and completely covered by the undulating membrane in short stumpy forms.

Slender trypanosomes were isolated on the 4th day, intermediates on the 5th or 6th day and stumpy trypanosomes on the 7th or 8th day of infection. Long slender and short stumpy forms obtained in this way are contaminated with less than 5% of the other forms (33).

Trypanosomes were isolated from blood cells by DEAE chromatography in phosphate-buffered glucose (34). Procyclic trypanosomes were cultured as described (35) and collected by centrifugation.

Enzymes and radioactive biochemicals

Restriction enzymes were purchased from Bethesda Research Laboratories and New England Biolabs and polynucleotide kinase from Boehringer Mannheim. All enzymes were used according to standard procedures (36) or as recommended by the supplier. Radioactive biochemicals were obtained from Amersham.

DNA and RNA isolation

Trypanosome DNA was isolated by standard techniques involving lysis by 0.5% (w/v) SDS, RNase A (100μg/ml) treatment, proteinase K (100μg/ml) treatment followed by repeated phenol/chloroform extractions and finally, ethanol precipitation.

RNA was isolated as described (37) and polyA$^+$-enriched RNA was obtained by oligo-dT-cellulose chromatography (36).

DNA and RNA blot and hybridization procedures

Restriction enzyme digests of trypanosome DNA were size fractionated on 0.7% (w/v) agarose gels made in 1×TBE (90 mM Tris, 90 mM H_3BO_4, 2 mM EDTA, pH 8.3). After electrophoresis, Southern blotting onto a Hybond-N nylon membrane (Amersham) was performed as described (36).

For field-inversion gel electrophoresis, trypanosomes were proteinase K digested in agarose blocks as described (38). Native DNA was separated in a conventional gel electrophoresis unit with a Chromopulse switcher (Amstelstad). Gels were run at 45 V in 1×TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.3) at 12°C. The switch time was 1500 s linearly increasing to 2918 s over a total run time of 118.20 hours. Southern blotting of the gel was as for conventional gels.

PolyA$^+$ RNA was denatured in the presence of glyoxal and DMSO and run on 1.5% (w/v) agarose with 10 mM NaH_2PO_4 (pH 7.0) as described (36). The RNA was blotted onto nitrocellulose BA85 (Schleicher and Schuell). BRL RNA size standards were used to estimate the sizes of hybridizing transcripts.

Filters were prehybridized and hybridized as described (36). Probes were radioactively labelled by random priming (Amersham).

Cloning Procedure

Following the considerations of Lathe (39), a *T.brucei* codon usage table was compiled from sequences available in the EMBL data bank. Only one VSG gene was included in the compilation. The table was used to design a single 56-mer oligonucleotide of the sequence: 5′-CTAACTCCATGTATGGTTGTCTGGGCG-ACAGCAGGCTCTGAAGCTGACTGGATATT-3′. The corresponding amino acid sequence is derived from a comparison between the region III conserved sequences of human polα and yeast POL1 (figure 2). The 56-mer was phosphorylated using polynucleotide kinase and γ-^{32}P-ATP and used as a hybridization probe to screen a genomic *T.brucei* 427 library in the phage lambda derived EMBL3 vector (26). This library was a gift from A.W.C.A. Cornelissen. Plaques were lifted in duplicate onto nitrocellulose (BA85, Schleicher and Schuell) and filters were hybridized with the end-labelled 56-mer at 45°C in 5×Denhardt's, 1 M NaCl, 0.5% (w/v) SDS, 10% (w/v) dextran sulfate, 10 mM $Na_4P_2O_7$ and 0.2 mg/ml denatured salmon sperm DNA.

DNA sequencing

Restriction enzyme fragments from clones EMBLtbrDpol1 and 6 were subcloned in the M13 vectors mp18 and mp19 and sequenced by the dideoxy chain termination method (40) using modified T7 DNA polymerase (USB). Both strands of the extended DNA region of figure 1A were sequenced at least once in overlapping stretches. To resolve compression artifacts dITP was used in the reactions instead of dGTP.

DNA sequences were analyzed with the computer programs Microgenie (Beckman) or DNASIS/PROSIS (Hitachi).

RESULTS

Cloning and sequence analysis of the *T.brucei* DNA polymerase α gene

A comparison of the amino acid sequences of human and yeast DNA polymerases α shows a 95% identity between human residues 943–961 and yeast residues 937–955, which are part

of region III (21, 22). We assumed that a putative trypanosome DNA polymerase α gene would also show a high degree of conservation in this domain and a 56-mer oligonucleotide corresponding to this region was synthesized. In Southern blot experiments the 56-mer probe hybridized with a single band at a washing stringency of 3×SSC, 55°C. A genomic library of *T.brucei* DNA in phage EMBL3 (26) was screened and 5 independent clones of the corresponding DNA region were obtained (figure 1A).

DNA sequencing of clone EMBLtbrDpol1 revealed two closely linked ORF's of 442 and 1339 codons (figure 1B). The 56-mer is 75% identical to the indicated fragment of the larger ORF and the corresponding peptide sequence has 17 residues in common with region III of both human and yeast DNA polymerases α (figure 2A). Further sequence comparison showed that the complete translation product contains the 6 conserved regions of replicative DNA polymerases (figure 2A).

The *T.brucei* DNA polymerase shows a higher degree of similarity to human and yeast DNA polymerase α than to the DNA polymerase δ of yeast (figure 2A + B). We conclude that the cloned gene represents the *T.brucei* polα homologue.

Apart from the six regions identified earlier, an additional seventh conserved region of 23 residues can be distinguished between regions I and V (figure 2A, region VII). The DNA

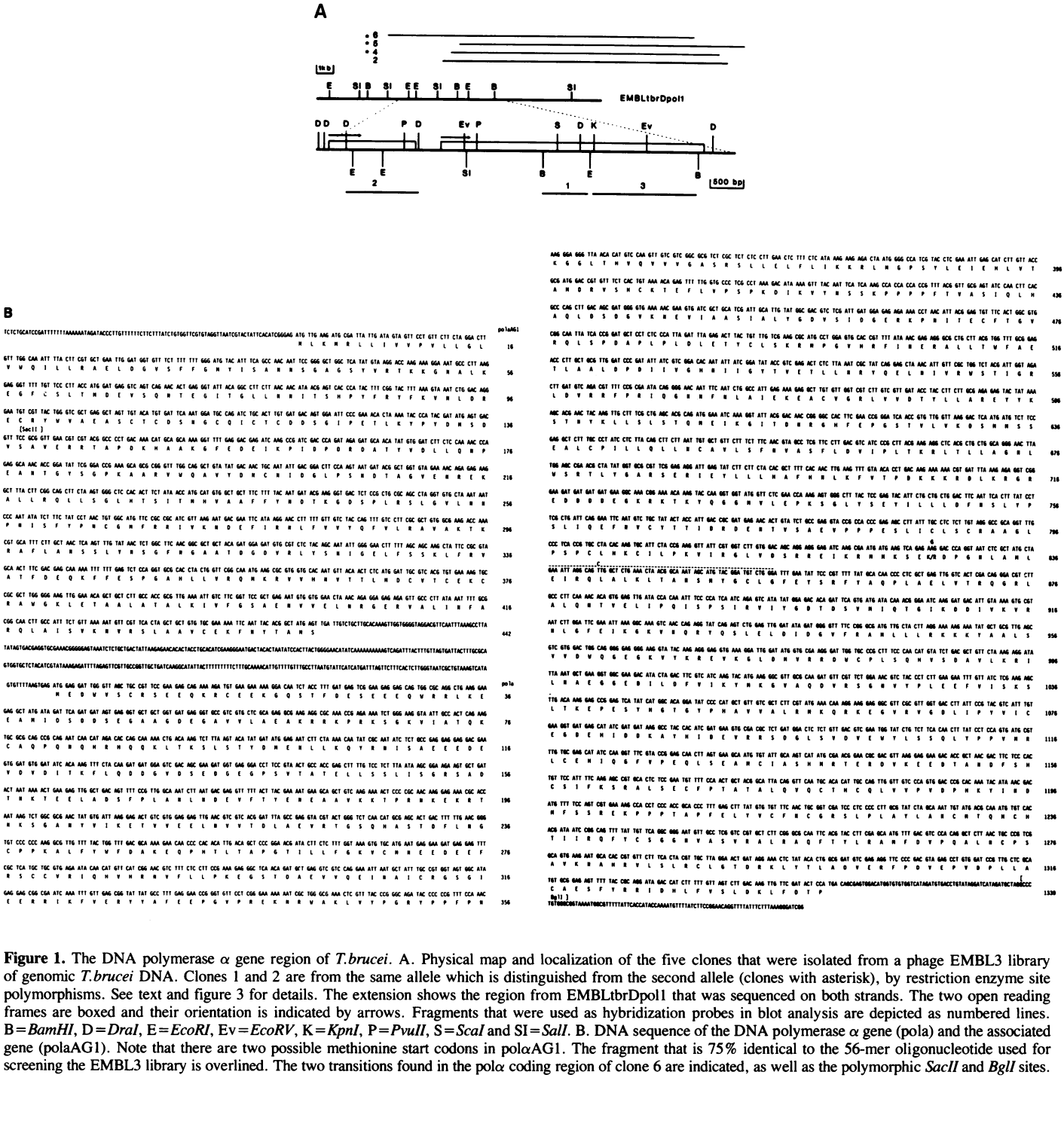

Figure 1. The DNA polymerase α gene region of *T.brucei*. A. Physical map and localization of the five clones that were isolated from a phage EMBL3 library of genomic *T.brucei* DNA. Clones 1 and 2 are from the same allele which is distinguished from the second allele (clones with asterisk), by restriction enzyme site polymorphisms. See text and figure 3 for details. The extension shows the region from EMBLtbrDpol1 that was sequenced on both strands. The two open reading frames are boxed and their orientation is indicated by arrows. Fragments that were used as hybridization probes in blot analysis are depicted as numbered lines. B=*BamHI*, D=*DraI*, E=*EcoRI*, Ev=*EcoRV*, K=*KpnI*, P=*PvuII*, S=*ScaI* and SI=*SalI*. B. DNA sequence of the DNA polymerase α gene (pola) and the associated gene (polaAG1). Note that there are two possible methionine start codons in polαAG1. The fragment that is 75% identical to the 56-mer oligonucleotide used for screening the EMBL3 library is overlined. The two transitions found in the polα coding region of clone 6 are indicated, as well as the polymorphic *SacII* and *BglI* sites.

polymerases encoded by EBV, CMV, HSV, as well as yeast DNA polymerase δ, show little similarity in this region; therefore, it would appear to be specifically conserved only in eukaryotic DNA polymerase α.

The primary structure of human and yeast DNA polymerases α differ in length by only 6 amino acids (1462 and 1468 aa, respectively). Interestingly, the *T.brucei* DNA polymerase α catalytic core is 123−129 amino acids shorter; the size difference is due to truncation in the N-terminus of the protein (figure 2B).

The linked ORF, which we named 'the polα associated gene 1' or polαAG1, codes for a protein with a calculated mol.wt. of 49,000 Da. Computer searches of the GenBank database (version R66.0) did not reveal significant homology to any previously characterized gene.

The polα genotype of *T.brucei*

In Southern blot experiments a probe for polα hybridizes with single *EcoRI* and *BamHI* fragments of *T.brucei* DNA (figure 3A, lanes 1 and 2) and fragment sizes correspond with those determined from the DNA sequence. Digestion with *PvuII* results in two equally strong bands (figure 3A, lane 3) which is the result of a *PvuII* polymorphism within the coding region of *T.brucei* polα. Three of the five clones examined were found to possess the additional *PvuII* site (see figure 1A) and clone 6 was sequenced around this site. The *PvuII* polymorphism is the result of a silent mutation of a thymine to a cytosine, coincidentally located in the region homologous to the 56-mer oligonucleotide used to isolate the clones (figure 1B). Closely linked to this site another polymorphism, adenine substituted for guanine, results in the conservative substitution of lysine for arginine at position 828 in the amino acid sequence (figure 1B), just outside conserved region VI.

Additional restriction site polymorphisms are detected for *EcoRV*, *KpnI*, *ScaI* (figure 3A, lanes 4−6), *BglI*, *XhoI* and *SacII* (not shown). One polymorphic *SacII* site is located within polαAG1 and this site is present in the sequenced clone, as is the polymorphic *BglI* site 3′ to polα (figure 1B). It appears that most of the polymorphic restriction enzyme sites are located upstream of polαAG1 suggesting lower selective pressure against mutations in this area.

Two different size classes of chromosomes hybridize to the polα gene probe, as shown by field inversion gel electrophoresis (FIGE) and subsequent Southern blot analysis (figure 3B). The estimated sizes of the chromosomes are 3.4 and 3.9 Mb. The two bands do not display a 1:1 stoichiometry on either the ethidium bromide stained gel or the autoradiograph. This may be due to trapping of some of the larger chromosomes in the slot as has been observed previously (41).

Physical mapping of a 22 kb region surrounding the two polα copies, and comparisons of the gene region in different *T.brucei* stocks and clones, showed that the pattern of polymorphic sites varies and that genetic exchange between the two copies apparently occurs (not shown). These results suggest that the two copies of the gene are allelic and that the two differently sized,

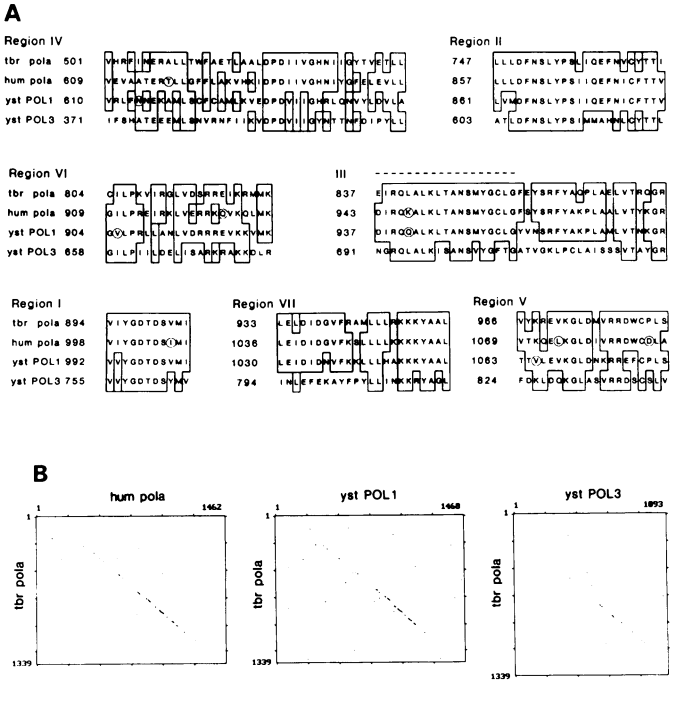

Figure 2. Comparison between eukaryotic replicative DNA polymerases. A. Conserved regions. The amino acid sequences are derived from *T.brucei* DNA polymerase α (tbr pola), human DNA polymerase α (hum pola) and *S. cerevisiae* DNA polymerases α (yst POL1) and δ (yst POL3). Amino acids that are identical in two or more DNA polymerases are boxed. Residues within blocks that are unique to a DNA polymerase are encircled for clarity. Numbering of the regions I-VI is according to Wong et al. (21). Region VII appears to be specifically conserved in α-type DNA polymerases. The overlined portion of region III formed the basis of the 56-mer oligonucleotide that was used to identify clones containing the *T.brucei* polα gene. B. Matrix comparisons of *T.brucei* DNA polymerase α to human and yeast DNA polymerases. A dot represents a match of 5 out of 8 amino acids.

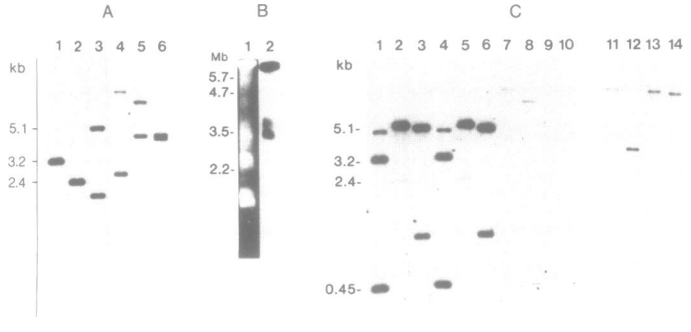

Figure 3. A. Polymorphic restriction sites in the genomic DNA region of *T.brucei* polα. Genomic *T.brucei* DNA was digested with the restriction enzymes indicated, size fractionated on a 0.7% w/v agarose gel and blotted onto a nylon membrane. The blot of lanes 1−3 was hybridized with polα probe 1 (figure 1A) and lanes 4−6 were hybridized with probe 2 from polαAG1. Filters were washed at 65°C, 0.1×SSC and autoradiographed. Restriction digest of the *T.brucei* genomic DNA are; lanes 1, *EcoRI*; 2, *BamHI*; 3, *PvuII*; 4, *EcoRV*; 5, *KpnI* and 6, *ScaI*. B. *T.brucei* chromosomes containing the polα loci. Lane 1 shows an ethidium bromide stained gel of *T.brucei* megabase sized chromosomal DNA molecules separated by FIGE. The DNA was blotted onto a nylon filter and hybridized with ^{32}P-labelled probe 1 from polα followed by washing at a stringency of 0.1×SSC at 65°C. Lane 2 shows the resultant autoradiogram. As size markers the *Saccharomyces cerevisiae* YNN295 chromosome of 2.2 Mb (Biorad) and *Schizosaccharomyces pombe* 927 chromosomes of 3.5, 4.7 and 5.7 Mb (FMC) were used. C. Conservation of the polα gene region among different trypanosomes. *T.b.brucei* (lanes 1−3), *T.b. rhodesiense* (lanes 4−6), *T.congolense* (lanes 7, 8, 11 and 12) and *T.vivax* (lanes 9, 10, 13 and 14) DNAs were digested with restriction enzymes and analyzed by Southern blot hybridization. The filter of lanes 1−10 was hybridized with probe 2 from polαAG1 of *T.brucei* (figure 1A). Probe 3 from polα was used for lanes 11−14. Hybridization was performed at 55°C and the filters were washed at 65°C, 3×SSC. The DNAs of lanes 1, 4, 7, 9, 11 and 13 were digested with *EcoRI*, of lanes 2, 5, 8, 10, 12 and 14 with *BamHI* and of lanes 3 and 6 with *PvuII*.

hybridizing chromosomes are homologous. It has been reported previously that homologous trypanosome chromosomes of the megabase size range may have different mobilities in pulsed field gel electrophoresis systems (26, 41, 42).

Conservation of the polα gene region

Southern blot analysis was also utilized to study the conservation of the polα gene region among different Salivarian trypanosomes (figure 3C). As expected from their close relatedness (43), digests of *T.b.rhodesiense* DNA show the same hybridization pattern as digests of *T.b.brucei* DNA, when the polαAG1 probe was used (figure 3C, lanes 1–6). The region is less well conserved in *T.congolense* and *T.vivax*. Hybridization of the polα gene probe to *T.congolense* DNA is weak and the physical map differs between the two species (figure 3C, lanes 11 and 12). The polαAG1 probe also hybridizes weakly to *T.congolense* DNA (figure 3C, lanes 7 and 8). Interestingly, further Southern blot analyses indicate that polαAG1 and polα are linked and organized in the *T.congolense* genome as reported herein for *T.brucei* (not shown).

Similarly, single restriction fragments of *T.vivax* DNA hybridize to the *T.brucei* polα gene probe (figure 3C, lanes 13 and 14). Probe 2 from the central part of the gene hybridizes to the same region, confirming that it contains polα (not shown). Linkage of polα with polαAG1 in *T.vivax* could not be demonstrated in Southern blots due to the requirement to use very low stringency washes, which results in multiple hybridizing restriction fragments.

Expression of *T.brucei* polα and polαAG1 during development

To study expression of the *T.brucei* polα gene, Northern blot analysis was performed with polyA$^+$ enriched RNA isolated from slender (dividing bloodstream form), intermediate, stumpy (non-dividing bloodstream form) and procyclic (dividing insect form) trypanosomes. The blot was hybridized with the polα probe 1 (figure 4). The mature polα messenger is 4.3 kb in all the parasite forms investigated. Expression is most prominent in the rapidly dividing slender bloodstream form and the procyclic form (lanes 1 and 4 respectively). The amount of polα mRNA is lower in the transitional intermediate bloodstream form (lane 2) and little is present in the stumpy bloodstream form (lane 3). Clearly, the expression of the polα gene is positively correlated with trypanosome division. Likewise, human polα expression was found to be cell proliferation dependent (21).

Interestingly, unlike polα mRNA, the polαAG1 mRNA of 1.8 kb is present at similar levels in all the *T.brucei* forms investigated and appears to be expressed constitutively (figure 4). This conclusion was confirmed by several independent Northern blot analyses.

In separate experiments, polα probe 3 and polαAG1 hybridized not only to their respective transcripts of 4.3 kb and 1.8 kb, but also to a RNA larger than 10 kb (figure 4, lane 5). A probe generated for the mini-exon sequence, which is highly repetitive in the trypanosome genome (44), does not hybridize with the latter mRNA, thus excluding the possibility that this hybridization represents contaminating DNA in the RNA preparation.

The large RNA could represent a precursor molecule which encompasses polαAG1 RNA and polα RNA. Further work to define the complete transcription unit is required.

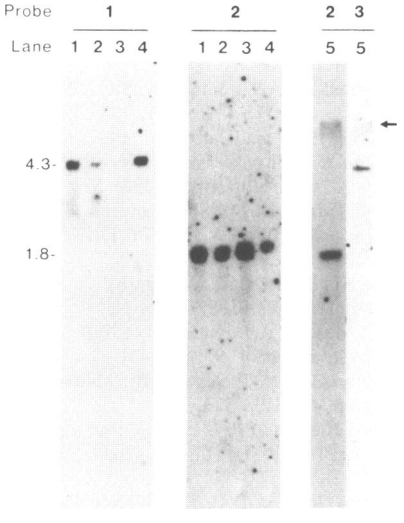

Figure 4. Expression of the polα gene region. PolyA$^+$ RNA was purified from long slender (lane 1), intermediate (lane 2) and stumpy (lane 3) bloodstream forms, and from *in vitro* propogated procyclic (lanes 4 and 5) forms of *T.brucei*. 3 μg of each RNA was size-fractionated on an agarose gel and blotted onto nitrocellulose. The filters were hybridized and rehybridized to the indicated probes, which were labelled by random priming. Probes 1 and 3 are derived from polα and probe 2 from polαAG1 (figure 1A). Filters were washed at 65°C, 0.1×SSC. After autoradiography, probes were removed by agitation in boiling water for 10 minutes. The arrow indicates a RNA species, larger than 10 kb, which contains both the polαAG1 and polα genes.

DISCUSSION

Conservation of the polα gene

Characterization of the proteins involved in DNA replication in a wide range of eukaryotic cells suggests that there is a high degree of conservation in their DNA replication complexes. This notion is supported by DNA sequence data for the genes encoding the core subunits of DNA polymerases α from man and yeast (21, 22). The sequence of the DNA polymerase α core of

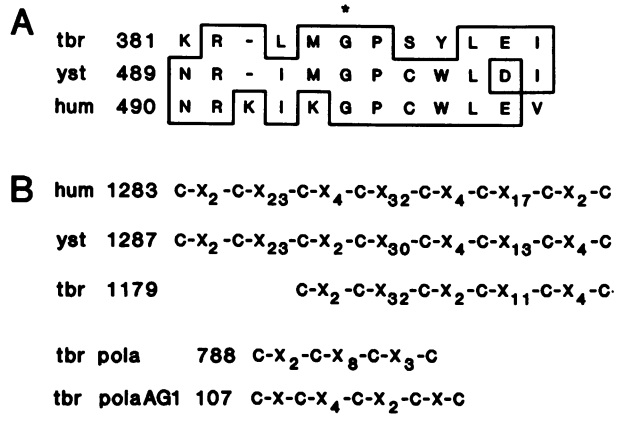

Figure 5. Putative functional domains in the *T.brucei* DNA polymerase α and the polαAG1 protein. A. Conserved domain in *T.brucei*, yeast and human DNA polymerases α (tbr, yst, and hum, respectively) which may interact with primase subunits. The asterisk indicates the glycine residue that is mutated to arginine in a yeast ts mutant resulting in apparent altered DNA polymerase α-primase stability (22, 49). B. Cysteine-rich sequences of *T.brucei* DNA polymerase α and the polαAG1 (polaAG1) protein which possibly form zinc-fingers. One of these structures, starting at position 1179 of *T.brucei* polα, appears to be conserved in the human and yeast DNA polymerases α at the same position relative to the 3'-end, and might be involved in DNA binding (50).

T.brucei, a protozoan only distantly related to both human and yeast, allows further evaluation of this homology.

The relationship among the α-like DNA polymerases is most evident from the conservation of seven regions that have virtually unaltered positions with regard to the C-terminal end of the three proteins examined (figure 2). Region VII, located between regions I and V, has not been previously identified. It appears to be conserved only in DNA polymerase α, but more data on DNA polymerase δ is needed to verify this.

Studies of mutant HSV DNA polymerase indicate that at least a portion of the conserved regions constitute important segments of the catalytic domains of the α-type DNA polymerases (25, 45–48).

Genetic studies indicate that the yeast DNA polymerase α residue, glycine 493, is in close contact with a primase subunit (22, 49). The glycine and its surrounding residues are also conserved in the human (50) and *T.brucei* (figure 5A) DNA polymerase α at approximately the same position with regard to the C-terminus, suggesting that in all three cases similar protein-protein interactions may take place between the core and the primase subunits.

Both the human and yeast DNA polymerases α possess a domain with closely spaced cysteine residues that may form DNA-binding zinc-fingers (50, 51; see figure 5B). These domains are situated near the C-terminal end of the proteins. At a corresponding position, the *T.brucei* DNA polymerase α shows such a domain, however, three, instead of four, cysteine pairs are present (figure 5B). Interestingly, another cysteine cluster, starting at position 788 (figure 5B), is located between conserved regions II and VI, bordering region VI of the *T.brucei* polα gene. This cluster is not conserved in the human and yeast DNA polymerases.

Linkage of polα to polαAG1

Stable transcripts from polα are evident in actively dividing *T. brucei* cells and are minimally detectable in non-dividing cells. In contrast, polαAG1 expression seems to be constitutive in all the forms of the parasite investigated.

Control of the differential expression of polαAG1 and polα might occur at the posttranscriptional level, as appears to be the case with the phosphoglycerate gene cluster of trypanosomes (52). It is not excluded, however, that the region is transcribed from more than one promoter, each promoter being associated with expression of a single gene.

The conserved linkage of polαAG1 with polα in both *T.brucei* and *T.congolense* may underlie a functional relationship between both gene products. It is tempting to speculate that the product of the polαAG1 gene might play some role in DNA synthesis.

Interestingly, polαAG1 encodes a cysteine rich domain (figure 5B) which could function in DNA binding. Further work is needed to establish whether the polαAG1 gene product has DNA-binding capacity and to elucidate its possible function, if any, in DNA replication.

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