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Duplication and transcription of procyclin genes in *Trypanosoma brucei*

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ABSTRACT

The genes encoding procyclin, the major glycoprotein expressed on the surface of procyclic forms of *Trypanosoma brucei*, comprise a multigene family. It has previously been demonstrated that procyclin genes in cloned trypanosome strains from Kenya and Uganda show restriction fragment polymorphisms. A detailed study of the Kenyan strain 227 has revealed that procyclin genes are arranged in tandem at 3 distinct loci (Pro A, B and C) and that the polymorphism is due to the duplication of 1.3 kb in the Pro A locus, which has generated an additional procyclin gene. Northern blot analysis has shown that at least 2 loci are transcribed and that a minimum of 3 procyclin genes are expressed within a cloned line. The transcription of procyclin genes is resistant to 1mg ml^{-1} α-amanitin, whereas that of the 5′ flanking gene in the Pro A locus is sensitive. This observation suggests that the two genes form part of separate transcription units with a promoter between them.

INTRODUCTION

African trypanosomes in their insect vector, the tsetse fly, must contend with an environment which is radically different from the bloodstream of their mammalian hosts. The procyclic forms in the insect midgut and the bloodstream forms in the mammal are not only distinguishable by their morphology and metabolism (reviewed in 1, 2), but also possess different surface coats. The major surface antigen of procyclic forms of *Trypanosoma brucei* is procyclin (3), a glycoprotein whose dominant feature is an extensive glutamic acid-proline (EP) dipeptide repeat (4, 5). In contrast, bloodstream forms are covered by a dense surface coat composed of a single species of glycoprotein known as the variant surface glycoprotein (VSG) (6). Whereas the VSG coat varies antigenically, owing to the sequential expression of different genes during the course of infection, procyclin appears to be conserved between isolates (7, 8).

Studies on the differentiation of bloodstream forms to procyclic forms *in vitro* have shown that the induction of procyclin mRNA expression is rapid (9), and that the appearance of the protein on the trypanosome surface precedes the loss of the VSG coat. In the fly, procyclin can be detected in procyclic and epimastigote forms, but is absent from metacyclic forms which have reacquired surface VSG (7).

There are multiple copies of procyclin genes in the genome of *T. brucei* (4, 5, 10). Restriction fragment polymorphisms (RFLPs) have been observed in trypanosome clones from

different geographical locations and also in cloned bloodstream forms and the procyclic culture forms derived from them (4). RFLPs have frequently been described for VSG genes (11) where they are a consequence of both the telomeric location of many copies (12) and also their predeliction for recombination and duplication. The insensitivity of procyclin genes to Bal 31, however, suggests that they are not located near telomeres (4). Studies on procyclin gene organisation in the *T. brucei* isolate TREU 667 (5, 10) have revealed that they are encoded at two distinct loci, each containing a pair of genes. The coding sequences of three of these genes are highly conserved, differing principally in the length of the hexanucleotide repeat, although the 3′ untranslated regions display less homology. The polypeptide encoded by the fourth procyclin gene in this isolate lacks a glycosylation site and its repetitive domain consists of six tandem copies of the pentapeptide GPEET followed by three EP repeats (13). Although this gene is transcribed in the same stage-specific manner as other procyclin genes and the mRNA can be isolated from membrane-bound ribosomes, it has not yet been demonstrated whether the protein is expressed.

Our aims in studying the organisation of procyclin genes in the Kenyan strain 227 were to determine the basis for the polymorphism we have observed and to establish which procyclin genes were transcribed. Here we show that procyclin genes are tandemly repeated at three loci, at least two of which are transcribed, and that the difference between isolates from Kenya and Uganda is probably due to unequal crossing over between alleles, resulting in the duplication of a procyclin gene and its flanking sequences.

MATERIALS AND METHODS

Trypanosome stocks; isolation of DNA and RNA

The trypanosomes used in this study were bloodstream forms of *T. b. brucei* strains 227 (ILTat 1.21; ref. 14) and 427 (MITat 1.2; ref. 15) and procyclic forms derived from ILTat 1.21. In this case, no alteration in procyclin genes was observed following differentiation (4). Bloodstream forms were grown in rats and isolated by cardiac puncture. Procyclic forms were cultured at 27°C in SDM-79 medium containing 10% foetal bovine serum (16). DNA (17) and RNA (18) were isolated as described previously. Nuclear run-ons were performed as described by Carrington et al. (19).

Electrophoresis, blotting and hybridisation

The separation of DNA fragments on agarose gels and blotting onto nitrocellulose were performed using standard procedures (18). Hybridisation was carried out as described previously (19), except that oligonucleotides were hybridised at 37°C. Washing conditions are described in the figure legends. Plasmid probes were labelled with ^{32}P by nick translation (20); oligonucleotides were end-labelled with ^{32}P using polynucleotide kinase (21).

Isolation of genomic clones and construction of maps

The construction of a genomic library from ILTat 1.21 DNA in λEMBL 3 has been described (19). Genomic clones of procyclin were selected by hybridisation with the cDNA

clone pPRO2001 (4). Inserts were mapped by partial restriction digests (22) and the maps confirmed by complete digests. Heteroduplex mapping was performed as described (23).

Hybridisation probes

In addition to the full length procyclin cDNA clone pPRO2001 (see above), the following oligonucleotides were used: TO2, complementary to nucleotides 75-96 of pPRO2001, was used as a probe for the 5′ end of the gene. TO3, complementary to nucleotides 637-652 of pPRO2001 was used as a probe for the 3′ end of the gene. Three additional oligonucleotides which distinguished between the 3′ untranslated regions of different cDNA clones were also used: TO5 is complementary to nucleotides 476-495 of pPRO2001. TO4 is complementary to nucleotides 535-554 of the procyclin cDNA clone PARP-2 (5) and TO9 is complementary to nucleotides 518-537 of the cDNA PARP β-667 (10). The approximate locations of the various oligonucleotides are shown in Fig. 1.

DNA sequencing

DNA fragments in pUC19 were sequenced directly or subcloned into M13 mp18 and mp19 as described (24). DNA sequencing was performed by the dideoxynucleotide chain termination method of Sanger et al. (25) using universal primers (Biolabs 1211 and 1212) or the oligonucleotides described above. Deletion subclones were generated by Bal 31 digestion using standard procedures.

RESULTS

Procyclin genes are present at three loci

Southern blot analysis was performed using genomic DNA from trypanosomes clones derived from strain 227 (ILTat 1.21) and strain 427 (MITat 1.2) digested with Hinf I. Hybridisation with pPRO2001, a full length cDNA clone of procyclin, revealed fragments of 4.3kb (Pro A), 3.0kb (Pro B) and 2.2kb (Pro C) in ILTat 1.21 (Fig. 2). In contrast, the 4.3kb fragment was not detected in MITat 1.2 DNA.

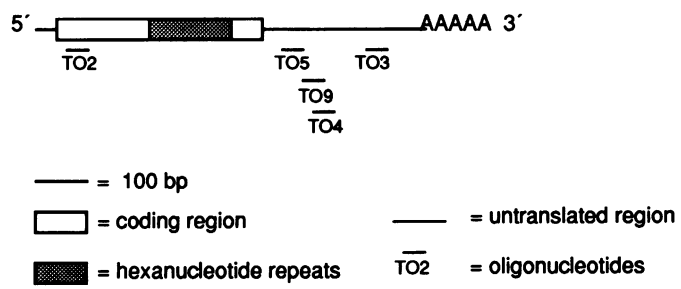

Fig. 1. Schematic representation of a procyclin cDNA clone with the approximate location of the oligonucleotides used in this study. The sequences and exact locations are given in Materials and Methods.

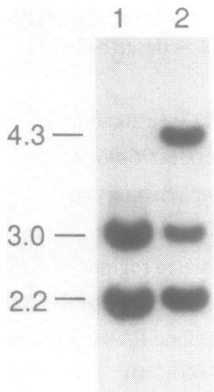

Fig. 2. Southern blot analysis reveals RFLPs in different strains of *T. brucei*. Genomic DNA from the Ugandan strain 427 (MITat 1.2; lane 1) and the Kenyan strain 227 (ILTat 1.21; lane 2) was digested with Hinf I. Electrophoretic separation and Southern blotting were performed as described previously (4). The blot was probed with nick translated pPRO2001 (see Materials and Methods). After hybridisation, the blot was washed with 0.1 x SSC, 0.1% SDS at 65°C.

Genomic clones containing each of the 3 fragments were isolated from a library of ILTat 1.21 DNA in λEMBL3 and restriction maps constructed. Fig. 3a shows the restriction enzyme maps of clones of the Pro A, B and C loci, respectively, with the location of the procyclin gene copies indicated. By the criteria of restriction enzyme mapping and heteroduplex analysis (Fig. 3b) the Pro A and B clones show homology over a length of 7.7 kb, extending from the 5′ border of Pro B. In Pro A, however, this region of homology is interrupted by a stretch of 1.3kb which is flanked by Pvu II sites. Further downstream, the restriction maps of the two clones diverge, although Southern blot analysis using specific restriction fragments as probes revealed the presence of cross-hybridising sequences (data not shown). The restriction map obtained for Pro C shows no obvious similarity to either Pro A or Pro B beyond the vicinity of the procyclin genes. The lack of homology between the flanking sequences in Pro C with those in either Pro A or B was confirmed by hybridisation experiments. These results, together with the relative intensities of hybridisation of the three fragments in genomic DNA from strain 227 (Fig. 2) make it likely that Pro A and B are alleles and that there are two copies of Pro C in the diploid genome. At present it is not known how closely the loci are linked, although pulse field gel electrophoresis has shown that all copies of procyclin are located on large chromosomes (4).

Southern blot analysis of Pvu II digests of each genomic clone revealed more than one hybridising fragment (Fig. 4), despite the absence of a Pvu II site in the procyclin cDNA probe. The intensity of hybridisation was similar for all fragments except a 5 kb fragment in Pro C which gave a weaker signal than the others. To establish whether these multiple bands were due to the presence of introns within a single procyclin gene at each locus, or to the presence of multiple copies of the gene, replicate filters were hybridised with an oligonucleotide complementary to a conserved sequence at the 5′ end of procyclin cDNAs (TO2; Fig. 4). The hybridisation pattern obtained with this oligonucleotide was identical to that obtained with the cDNA clone, suggesting that each Pvu II fragment contained at least the 5′ end of the gene.

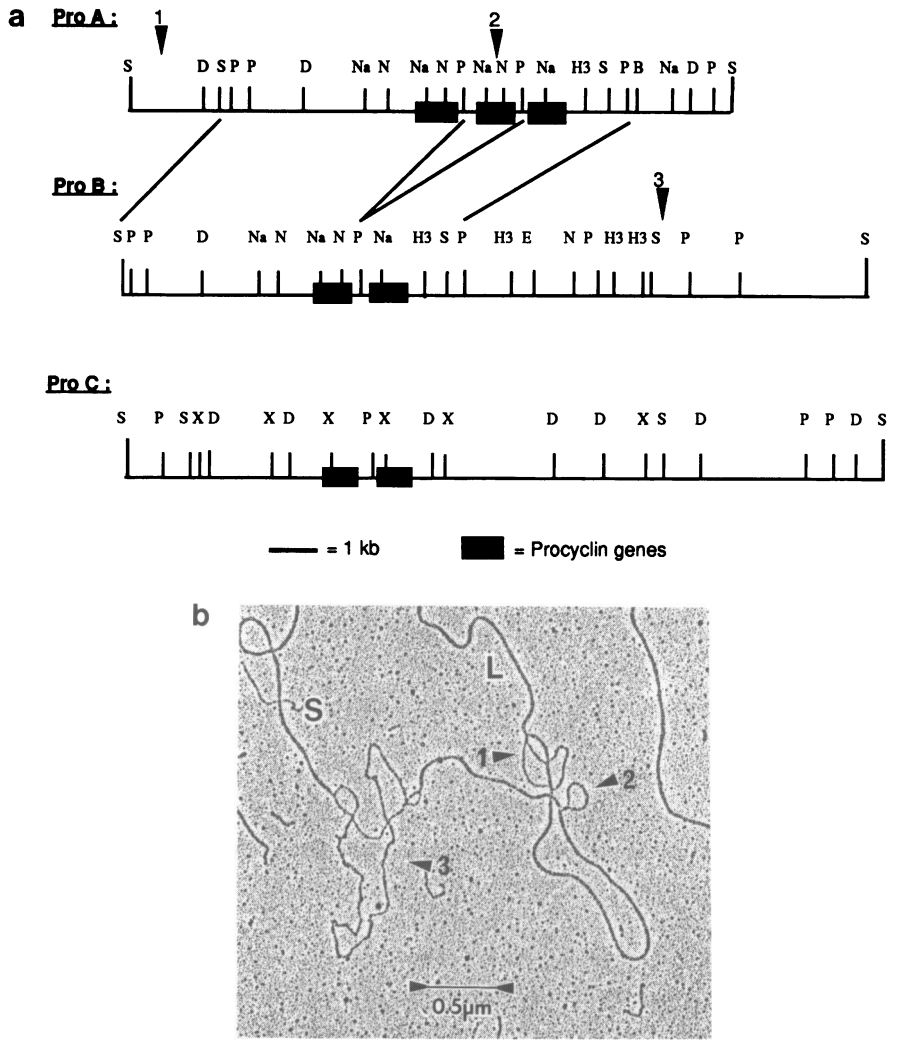

Fig. 3 (a). Restriction enzyme maps of genomic clones of Pro A, B and C. Sal I (S), Pvu II (P) and Dra I (D) sites were mapped in all three clones, Nar I (Na), Nde I (N), Hind III (H3) and Eco R1 (E) sites in Pro A and B only, Xmn I (X) sites in Pro C only. The maps are drawn so that the direction of transcription of procyclin genes is from left to right. (b) Heteroduplex formation between the genomic clones of Pro A and B depicted in (a). Arrowheads point to the three loops, "S" and "L" mark the short and long arms of the λ EMBL 3 vector.

Although the coding regions of different procyclin cDNA clones, derived from different trypanosome isolates, are highly conserved, the 3′ untranslated regions are considerably less so. Comparison of the first two cDNA clones to be sequenced (4, 5) showed only 40% homology between the 3′ untranslated regions. The most extended region of homology was a

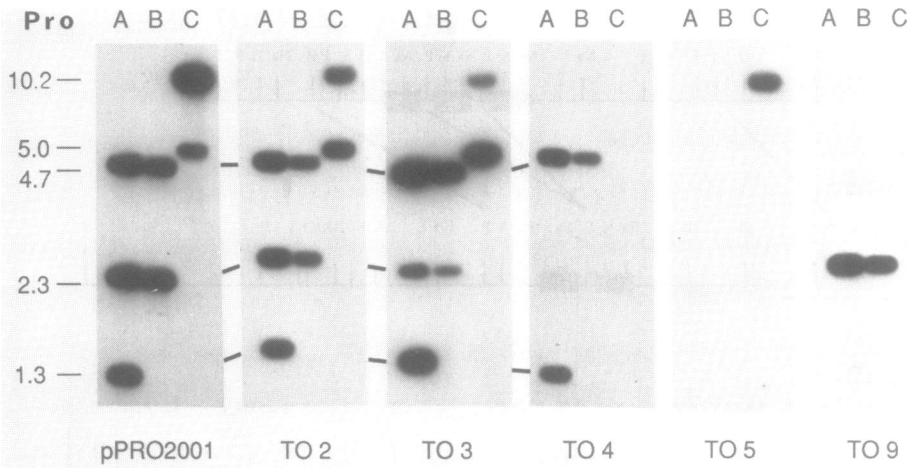

Fig. 4. Each genomic clone contains more than one copy of procyclin. DNA from genomic clones of Pro A, B and C was digested with Pvu II. Replicate Southern blots were probed with either pPRO2001 or the oligonucleotides shown (see Fig. 1). Following hybridisation, the blot probed with pPRO2001 was washed as described in the legend to Fig. 2. Blots probed with oligonucleotides were washed at 50°C in 3 x SSC, 0.1% SDS.

stretch of 16 nucleotides (including one pyrimidine exchange) located at position 637-652 in pPRO2001. An oligonucleotide complementary to this sequence (TO3) was therefore used as a probe for the 3′ untranslated region of the gene (Fig. 4) Once again the identical hybridisation pattern was observed, indicating that each fragment carried an entire copy of the gene. The differences in hybridisation intensity are probably due to single base mismatches within this sequence, as under the conditions used, no significant hybridisation could be seen using a 20-mer containing 2 mismatches (see below). Thus Pro A has three tandem copies of procyclin, including one within the 1.3kb insertion, and Pro B and C each have two copies.

To discriminate between different copies of procyclin and to locate them in the genome of strain 227, oligonucleotides were selected which hybridised specifically to non-conserved sequences in the 3′ untranslated regions of 3 different cDNA clones (4, 5, 10). The origins of oligonucleotides TO4, TO5 and TO9 are given in Materials and Methods. Under the conditions used, no significant cross-hybridisation was observed between the different genes. Three copies hybridised with TO4, one with TO5 and two with TO9 (Fig. 4). Thus all three different 3′ ends are present in the genome and account for six of the seven copies. The single exception is the copy on the 5kb Pvu II fragment in Pro C, although under conditions of lower stringency this fragment also bound TO4 despite 2 mismatches within the sequence (data not shown). Further sequence analysis revealed that this copy is equivalent to the PARP Aα gene described by Mowatt and Clayton (13), encoding tandem repeats of the pentapeptide GPEET. Apart from

two tandem copies in Pro A, which were both recognised by TO4, linked procyclin genes possessed different 3′ ends.

Transcription of procyclin genes

To establish whether more than one procyclin gene was being transcribed within a cloned line of procyclic form trypanosomes, the three 3′-specific oligonucleotides described above were used as probes on Northern blots of procyclic form RNA (Fig. 5). All three oligonucleotides hybridised to the RNA, consistent with the interpretation that several (and possibly all) procyclin genes are transcribed. In the absence of unique sequences for procyclin genes in either Pro A or B, it is impossible to say unequivocally whether one or both of these loci are active. It is clear, however, that the transcripts hybridising with TO5 must originate from Pro C. In keeping with the observation that the length of the repeat sequence varies between copies, the sizes of the transcripts recognised by the three oligonucleotides differ. It is notable that TO4, which potentially recognises three different transcripts, including one derived from the 1.3 kb insertion in Pro A, hybridises to a more diffuse band than either TO5 or TO9.

The effect of ∝-amanitin on the transcription of procyclin and a 5′ flanking gene was examined (Fig. 5b). The gene upstream of the procyclin genes in Pro A has recently been identified as a member of a family encoding microtubule-associated repetitive proteins (MARPs; refs. 26 & 27). In addition to procyclin and MARP, a clone containing part of a ribosomal gene was included as a control. Whereas transcription of the procyclin and ribosomal genes was resistant to 1mg ml^{-1} ∝-amanitin, that of MARP was completely inhibited. It thus appears that procyclin and MARP are transcribed by polymerases with different sensitivities to ∝-amanitin.

Origin of the 1.3kb insertion in Pro A

The Pvu II sites flanking the insertion in Pro A were exploited in cloning this region. The nucleotide sequence of the entire 1.3 kb fragment, deduced from the clone pAP3, is shown in Fig. 6. As predicted by hybridisation with the oligonucleotides specific for the 5′ and 3′ ends of procyclin, this fragment encodes a complete copy of a procyclin gene from nucleotides 343-1018. The sequence of this gene is virtually identical to that of the PARP-2 cDNA clone described by Mowatt and Clayton (5) with the exception of three point mutations (Fig. 6) and the fact that it contains 15 hexanucleotide repeats, rather than 29. By alignment of the two sequences it appears that deletions have occurred at two places within the repeat. Despite these changes the open reading frame has been preserved and the encoded polypeptide possesses all the characteristics of the procyclin precursor. These include the signal sequence, glycosylation site, an EP repeat and hydrophobic tail. The one point mutation occurring within the coding sequence does not alter the amino acid sequence. No other extended open reading frames were detected in either orientation within the flanking sequences, but an open reading frame of 297 bases beginning with ATG was identified from nucleotides 782-486 on the negative strand, and also found to be present in PARP-2. Whether this strand is actually transcribed has not been investigated.

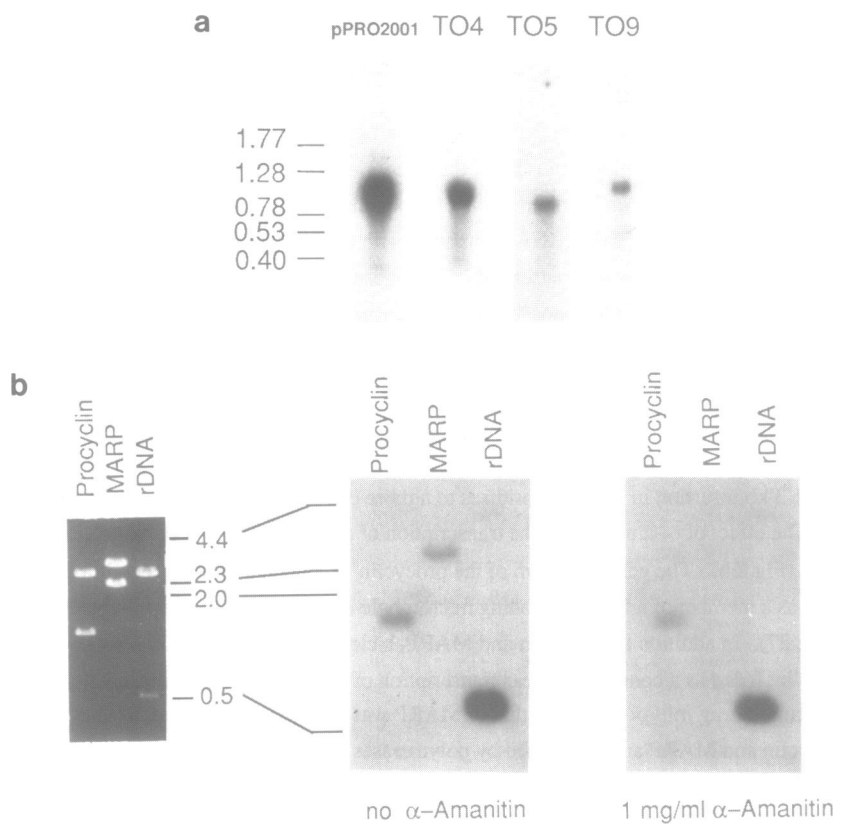

Fig. 5. (a) Several procyclin genes are transcribed. RNA was isolated from a cloned line (227.01) of *T. brucei* procyclic forms. One microgram of poly(A) RNA was loaded per track. Blots were hybridised with pPRO2001 or the oligonucleotides shown. Washing of the blots was performed as described in the legend to Fig. 4. (b) Effect of ∝-amanitin on transcription. ^{32}P-labelled RNA was synthesised in isolated nuclei with or without prior incubation with ∝-amanitin. The MARP clone (pAP1) contains the 5′ Sal I fragment from Pro A. The procyclin clone (pAP3) is described in the text and Fig. 6. In all restriction digests the upper band is the plasmid vector.

To compare the sequences flanking this copy with those of other procyclin genes, partial nucleotide sequences were determined. Sequencing was performed on plasmid subclones containing specific fragments using universal sequencing primers and the oligonucleotide TO2. The results are shown schematically in Fig. 6, with duplicated sequences depicted in matching shades. The extent of homology between duplicated regions exceded 98%; the remaining 2% were not unambiguously resolved as these sequences were determined from one strand only. These results show that the intergenic sequence present in Pro B is duplicated in Pro A, where it occurs on either side of the central procyclin gene. The simplest

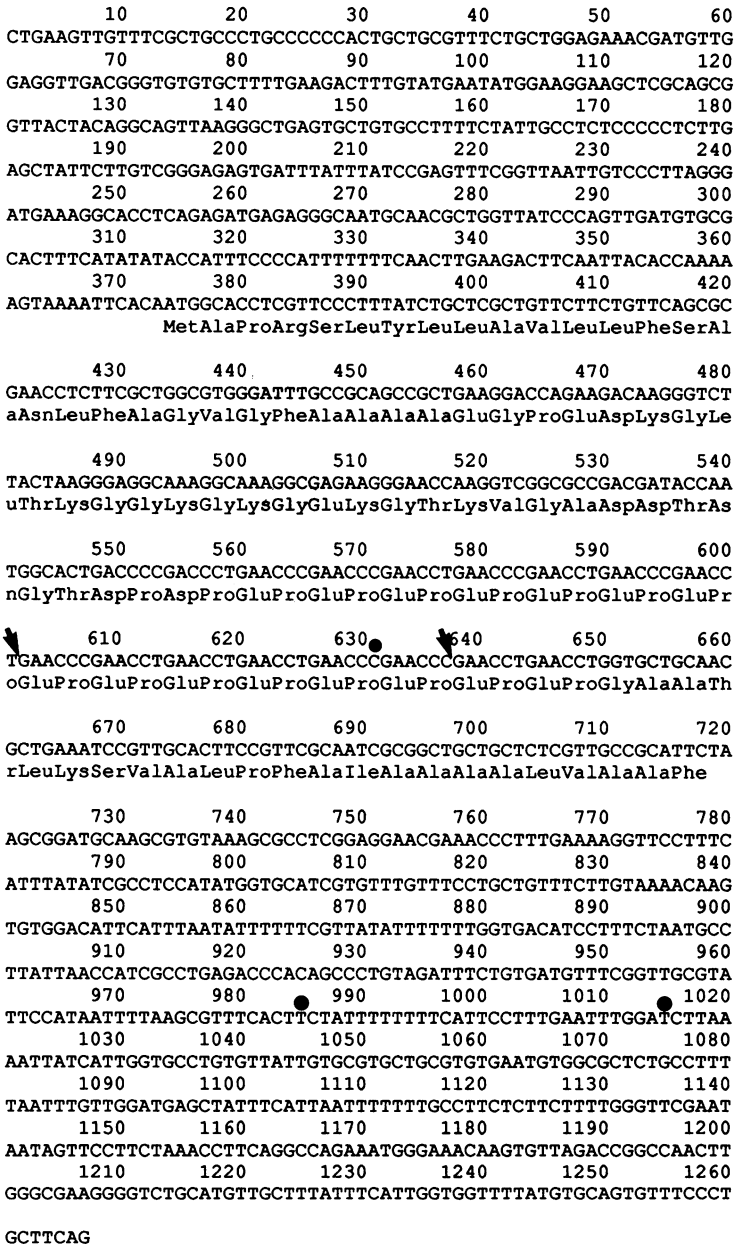

Fig. 6. Nucleotide sequence of the 1.3 kb insert in Pro A (pAP3; EMBL Data Bank Accession No. X16015). The entire sequence was determined in both orientations according to the method of Sanger et al. (25). Point mutations (●) and the sites of deletions (↘) are indicated. The amino acid sequence of the encoded procyclin polypeptide is shown below the DNA sequence.

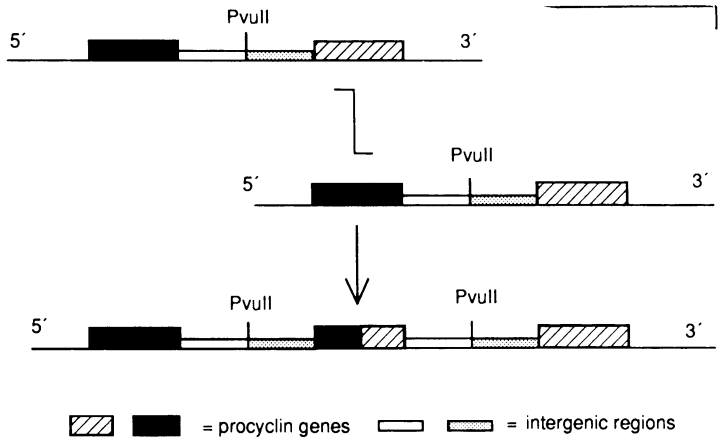

Fig. 7. Possible origin of the 1.3 kb insert in Pro A. Cross-over between the hexanucleotide repeats of procyclin genes in Pro A and B, leading to the duplication of intergenic sequences and the generation of a hybrid procyclin gene.

explanation consistent with all the data presented here is that the 1.3 kb insertion in Pro A was generated by unequal cross-over between repeats in a strain which formerly had two copies of procyclin in both Pro A and Pro B (Fig. 7), creating a new gene which is a hybrid.

DISCUSSION

An earlier comparison of *T. brucei* isolates from Kenya and Uganda revealed the presence of RFLPs in procyclin genes (4). In this study we have shown that the difference in restriction patterns is due to a duplication of 1.3 kb in the Pro A of the Kenyan strain 227, possibly through unequal cross-over between chromosomes. There is evidence that hybrid formation, and possibly conventional meiosis, occur during cyclical transmission of trypanosomes (28-31). Previous experiments have shown that cloning of bloodstream forms by syringe passage does not generate new RFLPs, although polymorphism was observed in procyclic forms derived from one of these bloodstream clones (4). These results would be consistent with rearrangements being confined to the insect stages of *T. brucei*.

The duplication in Pro A has given rise to an additional procyclin gene which has all the hallmarks of being functional, including flanking sequences derived from other, expressed, copies. The presence of these sequences make it feasible that correct trans-splicing and polyadenylation of the transcript would occur. The observation that a minimum of three copies of procyclin are transcribed within a cloned line agrees with the conclusions drawn from hybrid selection and *in vitro* translation (10). While it has not been established whether individual trypanosomes simultaneously express procyclin from more than one locus, our findings clearly demonstrate that at least two loci are capable of being transcribed.

There is evidence that a number of loci in the genome of *T. brucei* are organised as polycistronic transcription units which require subsequent processing of the primary transcript(s) to monocistronic mRNAs. The genes clustered within a transcription unit may have related functions, such as the alternating α- and β-tubulin genes (32), or may be coordinately expressed at specific life-cycle stages, such as VSG genes and their expression site-associated genes (ESAGs). The size of a single transcription unit may be extremely large, as is the case for a VSG expresson site encompassing 7 ESAGs, where the VSG gene lies 45kb downstream from the putative promoter (33). The gene upstream of the procyclin genes in Pro A is a member of the MARP gene family, encoding a protein with tandem repeats of 38 amino acids which are 50% homologous to the MARP repeat characterised by Schneider et al. (26). Two observations make it likely that procyclin and MARP belong to different transcription units. First, transcription of MARP is sensitive to α-amanitin while transcription of procyclin is resistant and second, expression of the MARP gene is not significantly different in bloodstream and procyclic forms (IR, unpublished). This raises the possiblity that a promoter lies between the MARP and procyclin genes. By analogy with the VSG expression site, procyclin genes may form part of a larger transcription unit containing other genes which are coordinately expressed. This possibility is currently being investigated.

Yet another possibility is that procyclin genes are constitutively expressed, and that regulation occurs post-transcriptionally. This might occur at the level of RNA processing (34) or by degradation of the mRNA. The regulation of stability via target sequences within the mRNA has been documented for a variety of genes ranging from ompA (35) and polynucleotide phosphorylase (36) in *E. coli* to the transferrin receptor (37), histone H3 (38) and the protooncogenes fos (39) and myc (40) in higher eukaryotes. We have shown here that the oligonucleotide TO3 recognises a consensus sequence of 16 nucleotides which is present in all procyclin genes. Sequence analysis of procyclin genes with different 3′ ends (4, 5, 10) has revealed that in all cases this sequence occurs at the same position, ending 76 bases upstream of the poly A addition site. Interestingly, a similar situation exists for the VSG genes of *T. brucei* where another consensus sequence of 16 nucleotides is found at the 3′ end of all VSG mRNAs (41-43). While no function has yet been ascribed to these sequences, it is conceivable that they contribute to the regulation of gene expression by affecting mRNA stability at different stages of the life cycle. This hypothesis, however, can first be tested when a reliable transfection system for trypanosomes becomes available.

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