Volume 12 Number 10 1984 Nucleic Acids Research

Modification of telomeric DNA in *Trypanosoma brucei;* a role in antigenic variation?

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Received 12 March 1984; Accepted 9 April 1984

ABSTRACT

Expression of surface antigen genes in Trypanosoma brucei occurs at expression sites located near telomeres. Since only one antigen is produced at a time, a mechanism must exist to prevent the simultaneous activity of multiple expression sites. Here we report that PstI and PvuII restriction sites in silent telomeric antigen genes are partially uncleavable, presumably as a consequence of DNA modification. The modification, which is absent in transcribed genes but returns after gene inactivation, may be specific for telomeric DNA because (1) it is not detected in non-telomeric genes; (2) modification is highest close to the telomere; (3) the level of modification in a telomeric gene is influenced by the size of the telomeric DNA segment downstream. Whether telomere modification is cause or consequence of antigen gene switch-off remains to be determined.

INTRODUCTION

Trypanosoma brucei and related species use 5 to 10% of their genome to specify about 1000 potential variant surface glycoprotein (VSG) genes (1). By the consecutive activation of genes out of this repertoire, trypanosomes change their VSG antigens and evade the immune response of the host (for review see refs. 2 and 3).

Expressed VSG genes have a telomeric chromosomal location. Most VSG genes are not normally located adjacent to a chromosome end and arrive at the telomere by a duplicative gene conversion, mediated by short homologous sequences at the edges of the duplicated segment (4-11). In eight different activation events of three non-telomeric VSG genes studied in our laboratory, the VSG gene duplicate was found to occupy the same telomeric expression site (12 and unpublished results). We have located this site on a 2000-kb chromosome and shown that activation of the VSG 118 gene involves an inter-chromosomal gene conversion (13). It remains to be proven whether the expression site provides a promoter for VSG

genes and encodes the common 5' 35 nucleotides of mature VSG mRNAs (14-17).

The presence of about 100 minichromosomes of 50 to 150-kb accounts for the large number of telomeric VSG genes in T.brucei (13). Activation of telomeric genes can proceed via a duplicative process similar to the one used by non-telomeric genes (18). Duplicative activation of the telomeric gene for VSG 221 yields a gene duplicate in the same expression site used by chromosome-internal genes (19). However, some telomeric genes can also be activated without concomitant gene duplication (20-21). The two modes of activation do not operate on two classes of VSG genes, because the single gene for VSG 221 can use both routes (19).

Our models explaining the tight intergenic exclusion between VSG genes were initially based on the assumption that the genome of T.brucei contains a single expression site for VSG genes (see 2 and 3). Genes were thought to enter this site by duplicative gene conversion or - hypothetically - by non-duplicative chromosome end exchange (see 3). However, using pulsed field gradient gel electrophoresis (22) to separate chromosome-sized DNA molecules of T.brucei, we have recently shown that non-duplicative activation of gene 221 does not involve its translocation to the 2000-kb expression site chromosome (13). T.brucei must therefore have more than one expression site for VSG genes. In the related species T.equiperdum at least three telomeric expression sites for surface antigen genes may be present (10). Because trypanosomes express only one VSG gene at a time, the presence of multiple VSG gene expression sites requires a mechanism controlling their activity.

Here we report that two silent telomeric VSG genes contain sites for the restriction enzymes PstI and PvuII that are only partially cleavable. We presume that the partial cleaving, which is not observed in expressed VSG genes, is due to DNA modification. We show that the modification is specific for telomeric DNA and discuss the implications for the mechanism of antigenic variation.

MATERIALS AND METHODS

Trypanosome clones derived from T.brucei, strain 427 were first described by Cross (23), and used in our laboratory to

infect rabbits as described in detail by Michels et al (12). Trypanosome clones were obtained from the blood of infected animals using the procedure of Van Meirvenne et al (24). The isolation of "first relapse" trypanosome populations by *in vitro* immune lysis with homologous antiserum has been described (19).

Trypanosomes were isolated from blood cells as described by Lanham (25). DNA was isolated, digested with restriction enzymes, size-fractionated and transferred to nitrocellulose according to published procedures (5, 26). To ensure complete digestion by PstI and PvuII, DNA was digested with a 10 to 20 fold excess of enzyme, purified by phenol extraction and ethanol precipitation and redigested as above. All data presented in this paper were obtained in multiple experiments with several independent DNA isolates. Blots were hybridized as described by Jeffreys and Flavell (27). DNA fragments of the VSG 221 cDNA clone TcV 221.5 (28; probe 1 from ref. 19) and the genomic DNA clone TgB 221.1 (1; probes 6, 7 and 8 from ref.19) were isolated as described by Girvitz et al. (29) and labeled by nick translation (30).

RESULTS

Partial cleaving of restriction sites in VSG gene 221

The single gene for VSG 221 in *T.brucei*, strain 427, is located adjacent to a chromosome end. Activation of this gene may occur either with concomitant gene duplication (in trypanosome clone 221b) or without (in clone 221a) (19). Maps of the 221 gene in trypanosome clones 221a and 221b, and in the VSG 118 producing clone 118a are presented in Figures 1A and 1B. Non-duplicative activation of gene 221 in clone 221a does not lead to DNA rearrangements within 50-kb upstream of the 221 coding region, other than a 0.15-kb insertion at the position indicated in Figure 1A. The duplicated 221b expression-linked copy (ELC) uses the same expression site as chromosome-internal VSG genes, is preferentially degraded during incubation of 221b nuclei with DNAase I, and is therefore probably the transcribed gene (19). The telomeric DNA segment downstream of gene 221 varies in length because of growth and deletion processes that presumably affect all telomeres of *T.brucei* (31, 32).

During our analysis of gene 221 we noticed that several PstI and PvuII restriction sites are only partially cleaved in the DNA

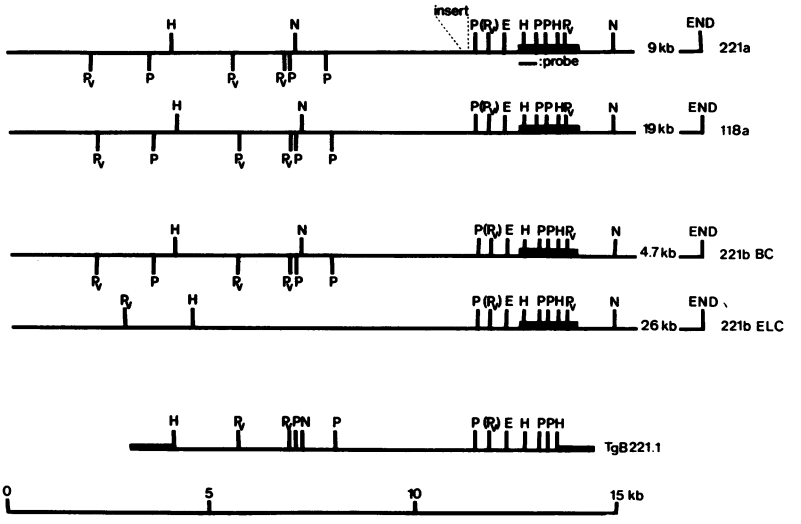

Fig.1; Physical maps of the gene(s) for VSG 221 in three trypanosome clones, and of the recombinant DNA clone TgB 221.1. (A); Map of the 221 gene in trypanosome clone 221a (top) and 118a (bottom). The two maps differ only in the length of the telomeric DNA segment downstream of gene 221, and in a 0.15-kb insertion upstream of the 221 coding region in clone 221a (see text). The size of the telomeric DNA segment measured between the downstream NarI site and the chromosome end, and the position of the 0.15-kb insert are indicated. The probe fragment used in the experiments reported here derives from the VSG 221 cDNA plasmid TcV 221.5 and maps upstream of the 5' PstI site in gene 221. (B); Map of the 221 basic copy (BC) and expression-linked copy (ELC) in trypanosome clone 221b. (C); Map of recombinant clone TgB 221 1 containing an upstream segment of gene 221 (1). Abbreviations of restriction enzymes: E: EcoRI; H: HindIII; P: PstI; Pv: PvuII; N: NarI; (Pv) marks two PvuII sites spaced 70 bp apart; END denotes the end of the chromosome. The sites drawn beneath the maps were localized with TgB 221.1 probe fragments.

of trypanosomes that do not express gene 221. This phenomenon is illustrated in the blots presented in Figures 2 and 3. In lane 1 of Figure 2, a 5' VSG 221 cDNA probe (see Fig.1A) is shown to hybridize to a single 1.9-kb fragment in a 221a DNA PvuII digest, as expected from the 221 gene map (Fig.1A). In a PvuII digest of DNA of trypanosome clone 118a, however, an extra - unexpected - fragment of 22-kb is observed (lane 2 of Fig.2). This additional fragment does not derive from a second, polymorphic copy of gene 221 in 118a, because gene quantitation indicates that clones 118a and 221a contain only a single copy of gene 221 per nucleus (19). The 22-kb fragment is also not the result of partial digestion of 118a DNA by PvuII, because over-digestion does not alter the

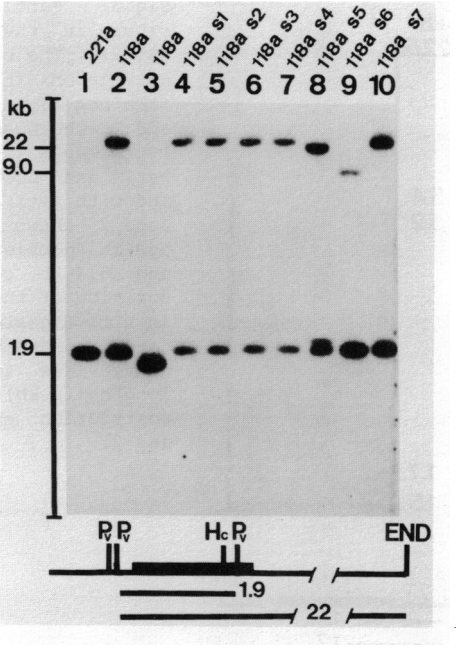

Fig.2; Partial cleaving of PvuII sites in the telomere containing the gene for VSG 221. Autoradiograph of a Southern blot of size-fractionated PvuII digests of trypanosome clone 221a DNA (lane 1) and 118a DNA (lane 2), a PvuII x HincII double digest of 118a DNA (lane 3) and PvuII digests of seven subclones derived from clone 118a (118as1-118as7; lanes 4-10 respectively). The blot was hybridized with a VSG 221 cDNA fragment (see Fig. 1A). The map beneath the blot shows the 221 gene in trypanosome clone 118a DNA and indicates the position of the hybridizing fragments (Hc: HincII; Pv: PvuII).

hybridisation pattern (not shown). The extra fragment maps between the PvuII site upstream of the coding region and the telomere downstream, so that a double digestion with PvuII and HincII shows a single fragment of 1.7-kb (lane 3, Fig.2).

To exclude the possibility that clone 118a is heterogeneous and consists of trypanosomes with and without a PvuII site in the 3' part of gene 221, we recloned 118a by inoculating single parasites into mice. Seven clones (118as1 - 118as7) were obtained, and all contained both the 1.9-kb and a larger fragment analogous to the 22-kb fragment in 118a (Fig.2, lanes 4-10). In 118as5 and 118as6 the extra fragment is shorter than 22-kb due to a deletion in the telomere downstream of gene 221. We have previously shown that deletions in telomeric DNA occur frequently and are a consequence of the trypanosome cloning procedure (31).

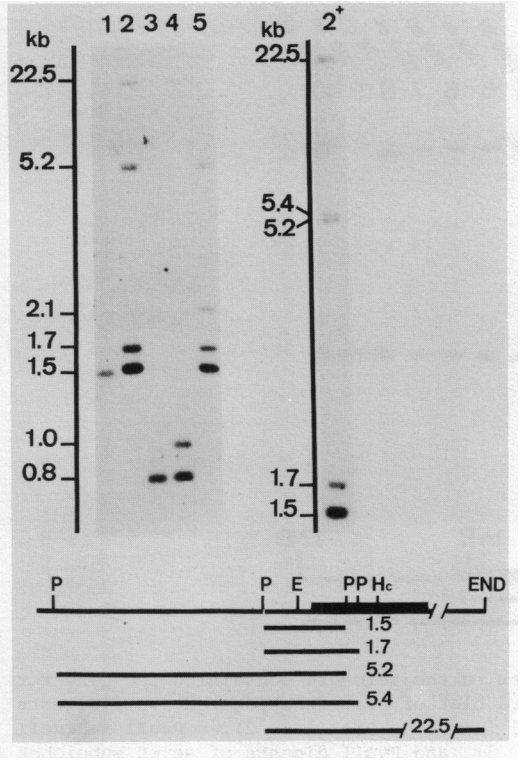

Fig.3; Partial cleaving of PstI sites in the telomeric gene for VSG 221. The blot contains digests of 221a DNA in lanes 1 and 3; and 2^+ 118a DNA digests in lanes 2, 2^+, 4 and 5. The DNAs were digested with PstI (lanes 1, 2 and 2^+); with PstI and EcoRI (lanes 3 and 4); and with PstI and HincII (lane 5). Lane 2^+ shows the resolution of the 5.2-kb doublet band into its 5.2 and 5.4-kb components. The map position of the fragments detected in PstI digested 118a DNA is indicated beneath the blot. The fragments are identified by their length (in kb). Abbreviations of restriction enzymes as in Figs. 1 and 2.

In 118as5 a third prominent fragment of 1.95-kb, which is only faintly visible in the remaining 6 clones and in the parent clone 118a, is detected. Double digests map this fragment between the PvuII site in the 3' part of gene 221 and a second upstream PvuII site, located 70-bp beyond the first upstream site (see map in Fig.2). Sequence analysis has confirmed the presence of both upstream sites (A.Bernards, unpublished).

We conclude from these data that the extra fragments detected in 118a derived clones are the result of a partial resistance to cleaving of the two 3'-most PvuII sites in the 221 telomere (see map beneath blot in Fig.2). We presume that these sites are partially modified in the inactive form of the 221 gene in trypanosome clone 118a and its derivatives. The prominent presence of the 1.95-kb fragment in 118as5, but not in the other clones, shows that the level of modification in different trypanosome clones is variable.

A similar situation is observed for three PstI sites in the

221 gene area, as shown in Figure 3. In a PstI digest of 221a DNA, which contains a transcribed 221 gene, only the expected 1.5-kb fragment is detected with a 5' cDNA probe (lane 1). However, the inactive 221 gene in DNA from trypanosome clone 118a yields extra fragments of 1.7, 5.2, 5.4 and 22-kb (lane 2 and 2^{+}), which are not lost by over-digestion or by recloning 118a (not shown).

The extra PstI fragments were mapped by conventional methods. Lanes 3 and 4 of Figure 3 show that EcoRI cuts the 1.5 and 1.7-kb fragments to 1.0 and 0.8-kb respectively, removes the 5.2/5.4-kb doublet, but not the 22-kb fragment. HincII does not cut the four shorter fragments but reduces the 22-kb fragment to 2.1-kb (lane 5). These and other double digests led to the mapping of the extra fragments in a PstI digest of 118a DNA as indicated beneath the map of the 221 gene in 118a DNA in Figure 3. We conclude that a single 221 gene can only produce these fragments if the three PstI sites which are closest to the telomere are partially uncleavable. Again we presume that DNA modification in the 221 telomere is responsible for this.

Expressed genes are not modified

In the previous section the expressed VSG gene in clone 221a showed no evidence of base modification while the silent 221 gene in clone 118a did. To test whether expressed genes are in general unmodified we analyzed trypanosome clone 221b, in which gene 221 is activated by duplicative transposition, so that both a silent 221 basic copy (BC) and an active expression-linked copy (ELC) are present (see ref. 19 and Fig. 1B). The results are presented in Figure 4, which also includes the analysis of two trypanosome populations derived from 221b (221br1 and 221br3) that do not express gene 221 anymore but retain the 221b ELC (Liu, A.Y.C., pers.comm.).

In clone 221b the silent 221 BC contains a modified PvuII site in the coding region, resulting in the 7.5-kb fragment observed in lane 1 of Figure 4 (see the map in Fig.1B). The ELC in 221b shows no evidence of PvuII site modification because no 28-kb fragment extending to the telomere downstream of the ELC is observed. In the two relapse populations on the other hand, both BC and ELC are modified, resulting in two "read-through" frag ments (lanes 2-3).

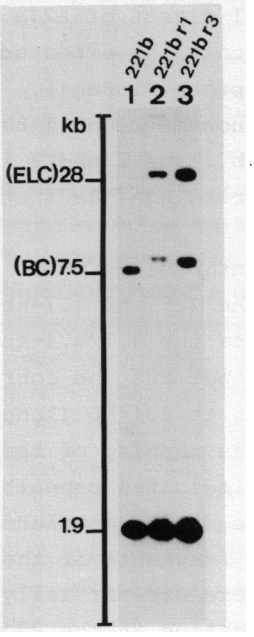

Fig.4; Partial cleaving of the 3' PvuII site in silent, but not in actively transcribed VSG 221 gene copies. The blot shows PvuII digests of trypanosome clone 221b DNA (lane 1), and of DNA from two trypanosome populations that have inactivated the 221 gene but retain the 221b ELC (221br1, lane2; 221br3, lane 3), and was hybridized with the VSG 221 cDNA fragment indicated in Fig. 1A. 221br1 and 221br3 were isolated by immune lysis of 10^6 221b trypansomes and amplification of the survivors in rats (19). Maps of the 221b BC and ELC are presented in Fig. 1B. The "read-through" fragment derived from the 221b BC is not of equal length in the three lanes due to growth of the 221 telomere during the isolation of the relapsed trypanosomes.

To further test whether inactivation of the 221 gene is coupled to a gain in modification, the behaviour of the gene was followed in additional relapse experiments. In four out of five relapses of trypanosome clone 221a, the inactivation of the 221 gene is accompanied by the complete loss of the gene and at least 8.5-kb of the upstream DNA segment, presumably because gene 221 has been replaced by another VSG gene. A single 221a relapse population, however, retains gene 221 after its inactivation (see ref. 19). The inactivated 221 gene in this relapse population has reacquired partially uncleavable PstI and PvuII sites (not shown). We conclude that the inactivation and modification of telomeric VSG genes are strictly correlated.

A specific modification of telomeric DNA

Three lines of evidence indicate that the downstream segment of telomeric DNA plays an important role in the modification of gene 221. First, over 50 PstI plus PvuII sites in the vicinity of ten non-telomeric VSG genes are completely cleaved in the DNA of all trypanosome clones tested (results not shown). Secondly, sites in the 221 gene close to the telomere are modified to a higher extent than distal sites. By scanning of autoradiographs

the modification level in 118a DNA was determined to be around 50% for the 3 sites in the coding region, and 5 or 15% for the upstream PvuII and PstI sites respectively. Similar gradients of modification exist in the 221 gene in several other trypanosome clones, although in rare cases the modification level of upstream sites approaches that of gene-internal sites.

The gradient of modification in gene 221 prompted us to use recombinant clone TgB 221.1 to analyze restriction sites located further upstream. Clone TgB 221.1 (see Fig. 1C) contains the 5' part of the 221 gene plus the adjacent 8.5 kb upstream, and was isolated from a HindIII clone bank of trypanosome clone 118a DNA (1). Using restriction fragments of this clone as hybridization probes three PstI and three PvuII sites were located upstream of the previously mapped area (indicated below the maps in Fig 1A). None of these sites shows evidence of partial cleaving in 118a or 221a DNA (results not shown), supporting our conclusion that only sites immediately adjacent to telomeres are affected.

More direct evidence for a role of the telomeric DNA in the modification of gene 221 is provided by the observation that genes with a long segment of telomeric DNA downstream are more highly modified than short-telomere genes. The relation between the size of a telomere and the modification level in the adjacent DNA segment is illustrated by Figure 2. This blot shows that six clones (lanes 4-8 and 10) derived from trypanosome clone 118a (lane 2) have a 221 telomere of similar size, whereas one 118a derived clone (lane 9) has lost 13-kb of telomeric DNA. The modification level of the 3' PvuII site in gene 221 follows from the ratio of the hybridisation intensities of the 1.9-kb and the larger "read-through" fragments, and is about 50% for the clones with a long 221 telomere but only 10% for the short telomere clone.

To investigate the correlation between length of a telomere and the level of modification of the adjacent DNA segment, we analyzed 23 trypanosome clones which do not express gene 221. The genealogy of the analyzed clones is presented in Table I. The length of the 221 telomere in these clones and the modification level of restriction sites in the 221 gene are given in Table II. Three conclusions can be drawn from the results summarized in Table II : (1) expressed genes are unmodified regardless of the

Day post infection	rabbit 1 (042)	rabbit 2 (118a)	rabbit 3 (1.152)	rabbit 4 (1.174)	rabbit 5 (121a)	rat 1 (118b)
8					1.2b-1(221b) 1.2b-2	
9		1.152 1.200 1.202 1.203 1.204				
14						1.205 1.206 1.207 1.208
18			1.172	1.5c(118c)		
20	1.6a(121a)	1.174	1.5b(118b)		1.4b(117b) 1.5d(118d)	
28	1.4a(117a) 1.5a(118a)					
36	1.6b(121b)					
45					1.194 1.196	

Table I; Genealogy of trypanosome clones in the MITat 1 serodeme (MITat = Molteno Institute Trypanozoon antigen type). Rabbit 1, infected with clone 042 has been described by Cross (23). Rabbits 2-5 were infected with 10^6 trypanosomes of the clone indicated in brackets (see ref. 12 for details). The rat was infected with 5 trypanosomes of clone 118b (Michels, P.A.M., unpublished experiment). If the VSG produced by a clone is known, the antigen type is specified (e.g. MITat 1.5a). In brackets are synonyms used in the literature (e.g. 118a). If the expressed VSG gene is not known, clone numbers are given (e.g. 1.152). Clones MITat 1.2b-1 and 1.2b-2 both produce VSG 221, both have activated the 221 gene via a duplicative proces and have indistinguishable 221 BC and ELC maps (unpublished data). Both clones therefore probably represent a single switching event. 221b in this paper and elsewhere (19) is synonymous with MITat 1.2b-1.

size of the downstream DNA segment; (2) growth of a telomere leads to an increased level of modification in the adjacent DNA segment; and (3) the modification is highest close to the telomere.

Preferential deletions in unmodified telomeres containing active genes

Since 221 genes with a short telomeric DNA segment downstream have a low level of modification, a deletion in the telomeric DNA segment should reduce the level of modification in gene 221. We have previously shown that deletions in telomeric DNA segments occur with low frequency both in vivo and in vitro during the manipulations required to isolate trypanosome clones. This latter effect can be mimicked by subjecting trypanosomes to a heat shock

Clone number	Length of 221 telomer (kb)	221 gene expressed?	Modification level 5'-PstI	3' PstI	3'PvuII
1.194	1.7	-	-	-	+/-
1.196	1.8	-	-	-	+/-
1.152	2.0	-	-	-	+/-
1.202	2.0	-	-	-	+/-
1.203	2.0	-	-	-	+/-
1.172	3.0	-	-	-	+
1.174	3.2	-	-	-	+/-
1.5b(118b)	3.2	-	-	-	+
1.5c(118c)	4.1	-	N.D	+	+
1.205	4.1	-	N.D	+	+
1.206	4.1	-	N.D	+	+
1.207	4.1	-	N.D	+	+
1.2b-1(221b)	4.7	-	+	+	+
1.2b-2	4.8	-	+/-	+/-	+
1.208	5.0	-	-	+/-	+
1.2a(221a)	9.0	+	-	-	-
1.1a	19.0	-	+	++	++
1.4a(117a)	19.0	-	+	++	++
1.5a(118a)	19.0	-	+	++	++
1.200	19.0	-	+	++	++
1.204	19.0	-	+	++	++
1.5d(118d)	24.0	-	++	++	++
1.6a(121a)	26.0	-	+	++	++
1.2b-1(221b)	26.0	+	-	-	-
1.2b-2	26.0	+	-	-	-
1.4b(117b)	26.0	-	+	++	++

Table II; Relation between the length of the telomeric DNA segment downstream of gene 221, and the level of modification of restriction sites in the adjacent DNA segment. For the derivation of the trypanosome clones see table I. Clone 221a was isolated during in vitro culture of clone 052 (12,23). The length of the 221 telomere is measured between the NarI site downstream of gene 221 (see fig. 1A) and the tip of the telomere. The sites analyzed are the PstI site upstream of the 221 coding region (5' PstI), and the two PstI and one PvuII sites in the coding region (3' PstI and 3' PvuII). The upstream PvuII site is slightly modified in trypanosome clones with 221 telomeres exceeding 19.0 kb in length only, and is not included in the table. Modification level: - : site completely cleaved; +/- : between 1 and 5% uncleaved; + : between 5 and 20% uncleaved; ++ : more than 20% uncleaved; N.D.: not determined.

(31). We therefore incubated 2500 trypanosomes of clone 118a (19-kb 221 telomere, high modification) in 0.1 ml guinea pig serum at 45°C and injected 0.01 ml aliquots taken after 0, 1, 2 and 3 minutes into mice. When the mice became parasitaemic after four to six days, trypanosomes were passaged on into rats for DNA isolation. The blot presented in Figure 5 shows that the 8-kb telomere downstream of the active 118a ELC is more likely to undergo deletions than the 19-kb telomere downstream of the silent 221 gene. The 25-kb telomere adjacent to the gene for VSG

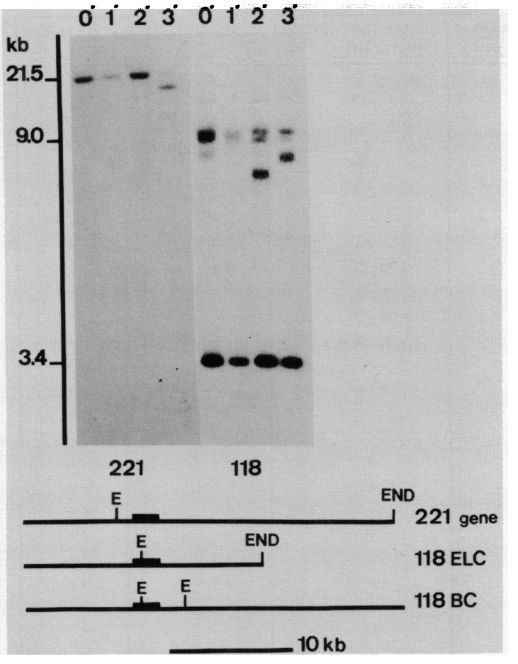

Fig.5; Southern blot showing preferential deletions upon heat shock in telomeric DNA segments downstream of actively transcribed VSG genes. Trypanosomes of clone 118a were incubated at 45° C for zero. one, two or three minutes prior to their injection into animals (see text). The blot contains EcoRI digested DNA of the control population (lane 0') and of the three heat shocked populations (lanes 1', 2' and 3'). The left panel of the blot was hybridized with a 5' VSG 221 cDNA fragment, the right panel with a fragment of the VSG 118 cDNA plasmid TcV 118.2 that maps downstream of the EcoRI site in the 118 gene (28).

1.8 in clone 118a (P.A.M. Michels, unpublished result) is even more resistant and does not undergo deletions even after three minutes of heat shock (not shown).

In a previous paper we showed that the 26-kb telomere downstream of the 221b ELC undergoes deletions when heat shocked whereas the 5.5-kb telomere downstream of the 221b BC was stable (31), and concluded that a greater length makes a telomere more vulnerable to deletions. However, the present results show that expression of a VSG gene increases the likelihood of deletions in the associated telomere, regardless of its size, as also recently reported by Pays et al. (33). Not surprisingly, the small decrease in size of the 221 telomere in trypanosomes which had received a 3 minute heat shock, is not correlated with an alteration in the level of 221 gene modification (not shown).

Modification of the VSG 118 ELC

Activation of the chromosome-internal gene for VSG 118 occurs by the integration of a duplicate gene in a telomeric expression site (12). We have been unable to detect partial cleaving of a PstI and a PvuII site present in the duplicated segment of the 118 gene in four trypanosome clones expressing this gene. Recently, we have cloned trypanosomes from first relapse popula-

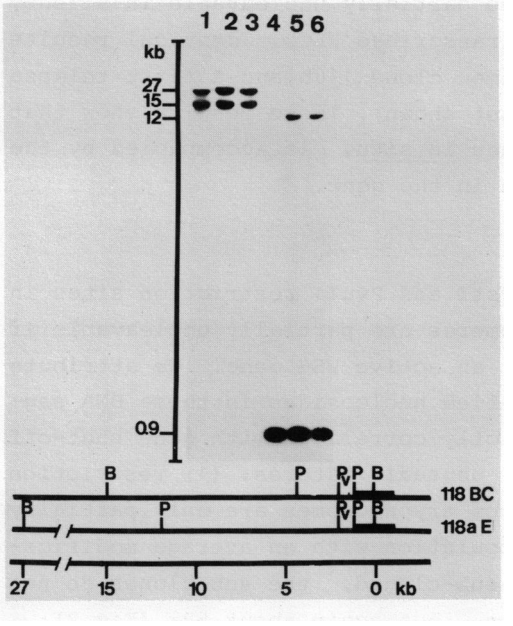

Fig.6; Partial cleaving of PstI in inactivated VSG 118 ELCs. The blot shows BamHI digests (lanes 1-3) and BamHI x PstI double digests (lanes 4-6) of trypanosome clone 118a DNA (lanes 1 and 4), and of two trypanosome clones derived from clone 118a which retain the 118 ELC in inactivated form (lanes 2,5 and 3,6 respectively). The BamHI and PstI maps of the relevant area of the VSG 118a BC and ELC are drawn beneath the blot. The cDNA fragment used as a probe derives from plasmid TcV 118.2 (28), and maps upstream of BamHI site in the VSG 118 gene.

tions of VSG 118 expressors, and found that some of these clones retain the 118 ELC in inactive form (3). To test our hypothesis that inactive telomeric VSG genes contain modified DNA, we have examined these clones for the presence of partially cleavable restriction sites in the silent 118 ELC.

A blot showing partial digestion of the PstI site in inactive 118 ELCs is presented in Figure 6. The map beneath the blot gives the positions of the relevant restriction sites in the 118a BC and ELC. The 118a ELC and its inactivated derivatives have identical restriction maps, at least up to the BamHI site at 26 kb upstream of the VSG 118 coding region (Michels, P.A.M., pers. comm.). The BamHI fragments hybridizing to a 5' 118 cDNA probe therefore comigrate at 15 kb (BC) and 27 kb (ELC) in lanes 1 to 3 of Figure 6. In 118a DNA, PstI cuts both fragments to 0.9 kb, as expected from the map (lane 4). However, in the two relapse clones the PstI site in the ELC is partially uncleavable, resulting in the sub-stoicheiometric 12 kb extra fragment in lanes 5 and 6. This interpretation was confirmed in triple digests (not shown).

A similar approach was used to analyze the PvuII site in the transposition unit of the 118 gene (see the map in Fig.6). Like

the PstI site, this site is also partially uncleavable in silent, but fully cleaved in actively transcribed ELCs. Identical results were obtained with trypanosome clone 118b and a first relapse clone retaining the 118b ELC (not shown). These results show that the inactivation of VSG genes in situ, is accompanied by the acquisition of DNA modification in the gene.

DISCUSSION

We show in this paper that PstI and PvuII restriction sites in DNA segments adjacent to telomeres are partially uncleavable if the telomere does not harbour an active VSG gene. We attribute this to the presence of modified nucleosides in these DNA segments. The modification is strictly correlated with gene shut-off and displays a number of highly unusual features: (1) restriction sites in a clonal population of trypanosomes are only partially modified. If a trypanosome population with an average modification of 50% at a given site is sub-cloned, the sub-clones do not contain 0% or 100% modification, but again about 50% (Fig.2); (2) PstI and PvuII sites located more than 1 kb upstream of the VSG 221 coding region and chromosome internal sites are not detectably modified; (3) growth of the 221 telomere is accompanied by an increased modification of the adjacent DNA. We have previously shown that most, if not all telomeres of T.brucei grow during trypanosome multiplication, presumably as a consequence of the replication of linear DNA molecules. Growth probably occurs through the addition of CCCTAA hexamers to the tip of telomeres (31, 32).

To explain the remarkable results reported here, we favour the hypothesis that modification results from the activity of an enzyme that recognizes telomeric structures or sequences, for instance CCCTAA repeats, and modifies adjacent DNA. We envisage the enzyme binding at telomeres and sliding down the DNA, each modifiable nucleoside having a low chance of being modified as the enzyme reaches it. The enzyme should not have a high preference for hemi-modified sites and it should dissociate from the DNA after the modification reaction. In this way telomeric DNA in dividing trypanosomes would remain partially modified at each site, with sites close to the telomere modified to a higher extent than distant sites. Although such an enzyme is hypotheti-

cal, analogous enzymes exist. An example is provided by the type I restriction enzymes, which bind at a specific sequence and cleave the DNA at one of many possible locations on either side (34).

In addition to the variations in modification level associated with an altered telomere size, we find slight variations in modification level which are not linked to telomeric size alterations (see Table II). These secondary variations could be due to differences in the trypanosome generation time or in the activity of the modifying enzyme in individual trypanosome clones, or to a (weak) preference for modification of already partially modified DNA.

The modified nucleoside in T.brucei telomeres has yet to be identified. However, we note that in T.equiperdum evidence has been obtained for the presence of an unknown dC-replacing pyrimidine. This modified nucleoside, which is not deoxymethylcytosine, is exclusively found in CsCl gradient fractions enriched in the DNA segment downstream of the telomeric Bordeaux Trypanozoon antigen type (BoTat) 1 gene (35). Whatever the nature of the modification in T.brucei telomeres, it only inhibits the cleaving by PstI (CTGCAG) and PvuII (CAGCTG), but not by any of the other restriction enzymes used to map the 221 gene (see 19).

If the function of this unusual type of modification is to distinguish telomeric DNA from other DNA, it is not clear why expressed VSG genes are not detectably modified, even when the segment of telomeric DNA downstream is very long. We therefore prefer the possibility that the modification of telomeric DNA controls the activity of telomeric VSG gene expression sites, by preventing VSG gene transcription. Since the 5' mini-exon and the promoter for VSG genes are not located anywhere near the coding region of expressed genes (16), whereas modification is limited to telomeric DNA, the shut off of VSG gene expression by DNA modification may be mediated by a change in chromatin structure rather than by a direct effect on promoter sequences.

If modification controls the expression of VSG genes, even a low level of modification suffices to inactivate genes, because none of the trypanosome clones with a low modification of gene 221 produce VSG 221. However, VSG genes may contain regulatory sites with a high affinity for the modifying enzyme (e.g the

adjacent CCCTAA tracts), which do not show the variation in modification level of the PstI and PvuII sites. In any event, the length variation of telomeres do not seem to play a role in the control of VSG gene expression, and only a complete loss of modification may render genes active.

The complete loss of modification required to activate a telomeric VSG gene might occur in either of two ways, depending on the forces that prevent modification of actively transcribed telomeres. 1) Telomere modification is prevented by attachment to a specific site in the nuclear matrix. After two rounds of DNA replication such a telomere generates unmodified daughters, in which the VSG gene is activated. Switching of VSG gene expression either occurs by transposing a new gene into an active telomere, or by displacement of the active telomere by another one at the nuclear matrix site. If there is only one site, only one VSG gene can be transcribed at a time. 2) Telomere modification is counter-acted by active transcription, which prevents access of the modifying enzyme to the telomere. Occasionally, however, the enzyme gets on and switches off the gene (like an unmodified phage DNA molecule occasionally sneaking through a bacterial host restriction system). Switch on of chromosome-internal VSG genes occurs through the integration of an unmodified duplicate into a telomeric expression site. To account for the activation of telomeric VSG genes we propose that gene duplication removes modification. Integration of the unmodified gene duplicate back into the telomere occupied by the modified parent gene, results in a VSG gene activation without alterations in the gene copy number. The small DNA rearrangement upstream of the 221 coding region, that we have previously found to be associated with the non-duplicative activation of gene 221 (19), may be a relic of such a reintegration.

In the last model for VSG gene control discussed here, activation and inactivation of genes are not necessarily coupled and, as a consequence, trypanosomes expressing more than one VSG gene at a time would arise. In an immunocompetent host these double expressors would never form a substantial fraction of the population. However, in the absence of immune selection double expressors will be more abundant, the fraction depending on the number of expression sites, the rate of switching of individual

sites, the rate of inactivation of telomeric genes and the number of trypanosome generations. We are currently screening trypanosomes grown for hundreds of generations without immune selection to test whether double expressors accumulate under these conditions.

Acknowledgements

We are grateful to Lex van der Ploeg, Titia de Lange, Paul Michels and Alvin Liu for stimulating discussions and to Wendy Gibson for reading the manuscript.

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