The EMBO Journal vol.4 no.4 pp.989–993, 1985

Inactivation and reactivation of a variant-specific antigen gene in cyclically transmitted *Trypanosoma brucei*

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Communicated by M.Steinert

In *Trypanosoma brucei*, the activation of the variant-specific antigen gene AnTat 1.1 proceeds by the synthesis of an additional gene copy, the AnTat 1.1 ELC, which is transposed to a new location, the expression site, where it is transcribed. Using the AnTat 1.1 variant to infect flies, we investigated the fate of the AnTat 1.1 ELC during cyclic transmission of *T. brucei*. We show here that the AnTat 1.1 ELC is conserved in procyclic trypanosomes, obtained either from the midgut of infected *Glossina* or from cultures, and in metacyclic trypanosomes, although the AnTat 1.1 serotype is not detected among metacyclic antigen types. This same AnTat 1.1 ELC, which is thus silent as the parasite develops in the insect vector, can be reactivated without duplication during the first parasitemia wave following cyclical transmission. This re-expression of the conserved ELC accounts for the early appearance of the 'ingested' antigenic type after passage through the fly.

Key words: cyclic transmission/DNA/gene/*Trypanosoma brucei*/variable antigen

Introduction

The life cycle of *Trypanosoma brucei brucei* consists of a sequence of developmental stages accompanied by profound morphological and biochemical alterations, including changes in the surface coat of the parasite. In the mammalian host, the bloodstream stage trypanosome is coated with a single species of glycoprotein, the variant surface glycoprotein (VSG) (Cross, 1975) that determines its antigen type. During chronic infection, a single clone of trypanosomes will, in a number of successive waves of parasitemia, express a vast repertoire of such variable antigen types (VATs) and thus escape the immune defence of its host. The expression of this repertoire is controlled at the transcriptional level, by the switching off and on of a collection of genes. At least two mechanisms allow surface antigen genes to be expressed: in the first one, the gene is duplicated, generating an additional copy, the expression linked copy or ELC (Hoeijmakers *et al.*, 1980; Agabian *et al.*, 1980; Pays *et al.*, 1981a), which is translocated to and transcribed in a telomeric expression site (De Lange and Borst, 1982; Raibaud *et al.*, 1983; Pays *et al.*, 1981b, 1982, 1983a; Laurent *et al.*, 1983). The ELC is generally lost when the trypanosome switches to the next antigen type (Pays *et al.*, 1983a). The second mechanism allows telomeric antigen genes to be expressed without apparent duplication (Young *et al.*, 1982; Pays *et al.*, 1983b; Bernards *et al.*, 1984). In this case however, the ELC of the preceding VAT is retained, although in an inactive form (Pays *et al.*, 1983b; Laurent *et al.*, 1984a; Buck *et al.*, 1984).

After their ingestion by a tsetse fly, trypanosomes first differentiate into non-infective procyclic forms, rapidly losing the glycoprotein coat characteristic of the bloodstream form (Seed, 1964; Brown *et al.*, 1973). Later, the parasites migrate from the midgut of the insect to its salivary glands, where they ultimately develop into metacyclic forms that re-acquire a surface coat as well as infectivity for the mammalian host. Only a small but characteristic fraction of the total repertoire of VATs is expressed at this stage (Le Ray *et al.*, 1978; Crowe *et al.*, 1983), regardless of the VAT used to infect the flies (Hajduk *et al.*, 1981). Although this 'ingested VAT' is not present in the metacyclic population (Hajduk *et al.*, 1981), it generally reappears early in the first parasitemia wave following cyclical transmission to mice (Hajduk and Vickerman, 1981), as if the parasite had kept the memory of the ingested VAT throughout its passage and development in the vector. The physical basis of this memory might be found in the inactivated ELC of the ingested VAT. This hypothesis is indeed suggested by the following observations: firstly, when bloodstream trypanosomes are induced to transform into procyclic forms by cultivation *in vitro*, a process which is thought to mimic their development in the midgut of the fly, the last expressed ELC is retained in the genome, although its transcription seems to be blocked (Overath *et al.*, 1983); secondly, when an ELC is retained in a silent form (ex-ELC) after a non-duplicative switch occurring in the bloodstream trypanosome, it appears to be preferentially reactivated (Laurent *et al.*, 1984b).

Further support for this hypothesis is provided here in studies of the fate of the AnTat 1.1 ELC after procyclic transformation of AnTat 1.1 trypanosomes in both *in vitro* and *in vivo* conditions.

Results

Clone transmission and derivation

As illustrated in Figure 1, tsetse flies were fed on AnTat 1.1D infected mice. After this first cyclic transmission, the 'post-fly' AnTat 1.1E variant was isolated and cloned. This variant was then used to infect other tsetse flies which were dissected to collect procyclic and metacyclic trypanosomes from the midgut and the salivary glands, respectively. The metacyclics were injected in mice to clone some of the early bloodstream variants, AnTat 1.30C, 1.1F and 1.9B. AnTat 1.30C was one of the metacyclic VATs (MVATs), while variants AnTat 1.1F and 1.9B were not found in the metacyclic population, but were detected within 9–10 days in the first wave of parasitemia, immediately after the appearance of MVATs in the bloodstream. We also transformed AnTat 1.1E bloodstream forms into procyclic forms by cultivation *in vitro*, in order to compare such culture forms with procyclic forms obtained *in vivo*.

In the three AnTat 1.1 homoisotypes, AnTat 1.1D, 1.1E and 1.1F, the same ELC is inserted into the same expression site

We analysed the AnTat 1.1 ELC in clones AnTat 1.1D, 1.1E and 1.1F. In AnTat 1.1D, the ELC is an almost complete copy of one of the members of the AnTat 1.1 multigene family, namely

the AnTat 1.1 basic copy (BC), also described as the '6.4-kb' sequence (Pays *et al.*, 1983b). As shown in Figure 2, this ELC does not contain a *Hind*III site located 204 bp upstream from the stop codon in the AnTat 1.1 BC and also present in the ELC of the AnTat 1.1A clone analysed previously (shown for comparison in Figure 2). Moreover, the restriction map of the AnTat 1.1D ELC is different from that of two other previously described AnTat 1.1 expressor clones, AnTat 1.1B and 1.1C (presence in the former of a 3′ *Sph*I site and absence of a 3′ *Msp*I site: see Figure 2 and Pays *et al.*, 1983b). The AnTat 1.1D ELC is thus most probably generated from the same basic copy but by a segmental gene conversion (Pays *et al.*, 1983a, 1983b) different from that observed in AnTat 1.1A, 1.1B and 1.1C.

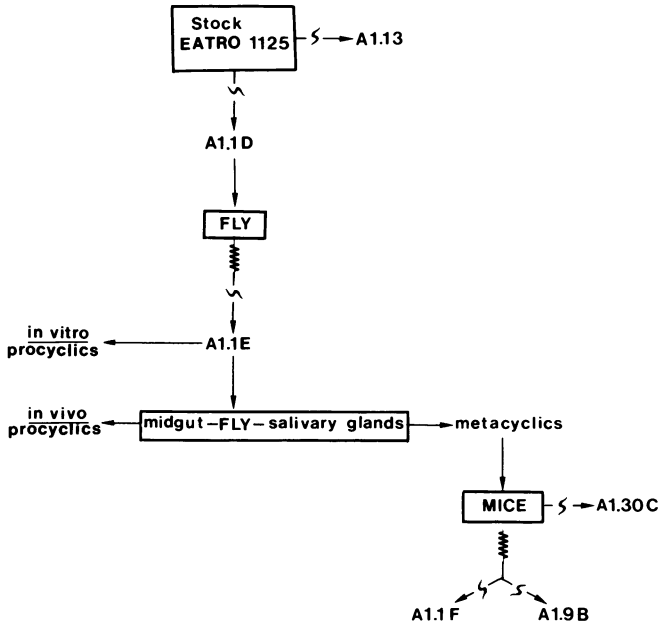

Fig. 1. Scheme of the cyclic transmissions and pedigree of the *T. brucei* clone populations referred to in this work. The symbols used are ⌇ for cloning and ⌇⌇⌇ for switch of antigenic type.

Interestingly, from their restriction maps (Figure 2), the ELCs present in AnTat 1.1E and 1.1F, the two homoisotypes isolated after cyclic transmissions through the fly, appear identical to the AnTat 1.1D ELC. In addition, in Southern blots with cDNA probes, the 5′ environment of the AnTat 1.1D, 1.1E and 1.1F ELCs appear to be identical, except for the size of the 5′ 'barren region', that region which is devoid of restriction sites. It is thus concluded that the same telomeric expression site has been used in these three homoisotypes. The same site was probably also used for the expression of the AnTat 1.1A (Figure 2) and 1.1B ELCs, but certainly not for ELC 1.1C (Pays *et al.*, 1983b).

The AnTat 1.1E ELC is conserved unaltered through cyclic transmission and retained by ensuing variants

The absence of any visible difference between the AnTat 1.1E and 1.1F ELCs strongly suggests that the AnTat 1.1E ELC was retained through cyclic transmission. This was further substantiated by comparative analyses of AnTat 1.1 specific sequences in six trypanosome populations whose relationships are given in Figure 1. A typical example is illustrated in Figure 3, where the presence of the same 21.5-kb *Pst*I fragment, which harbours the AnTat 1.1E ELC, indicates that the latter has been conserved in the culture procyclic forms derived *in vitro* from AnTat 1.1E, in the metacyclic clone AnTat 1.30C and in the two variants AnTat 1.1F and 1.9B, both cloned from the first wave of parasitemia after transmission.

Since it is not proven that procyclic trypanosomes obtained *in vitro* are totally identical with those developing naturally in the fly, we also, for the first time, analysed the DNA of pro-

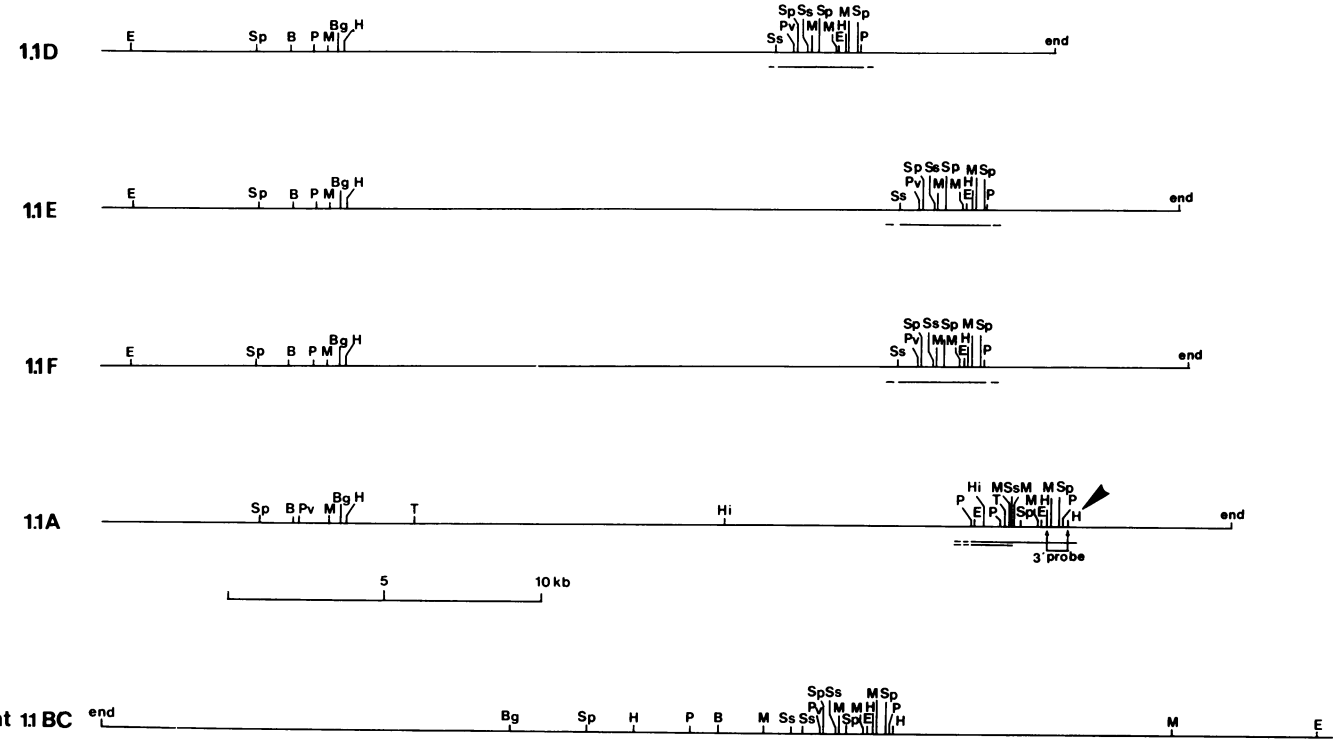

Fig. 2. Restriction map of the AnTat 1.1A, 1.1D, 1.1E and 1.1F ELCs and of the AnTat 1.1 basic copy (BC). The extent of the transposed element is indicated by the bar under each map, with uncertainties at the ends. The *Hind*III site indicated by an arrowhead is referred to in the text. Abbreviations used for restriction endonucleases are: B: *Bgl*I; Bg: *Bgl*II; E: *Eco*RI; H: *Hind*III; M: *Msp*; P: *Pst*I; Pv: *Pvu*II; Sp: *Sph*I; Ss: *Sst*I.

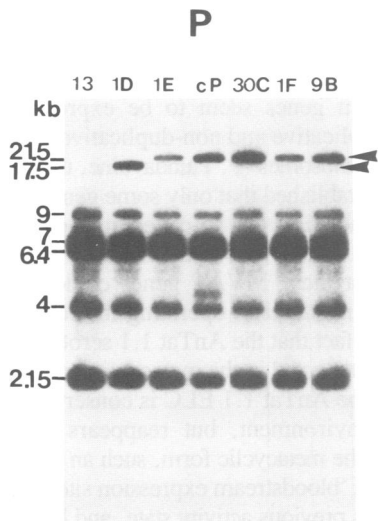

Fig. 3. Southern blot analysis of the AnTat 1.1 genes. From left to right, the DNAs analysed are from AnTat 1.13 (a non-expressor of the 1.1 gene), AnTat 1.1D, 1.1E, culture procyclics derived from AnTat 1.1E, AnTat 1.30C, 1.1F and 1.9B. Nuclear DNAs were digested with *Pst*I and hybridized with a probe made of the 550-bp *Hind*III-*Hind*III fragment of the AnTat 1.1A ELC (see AnTat 1.1A restriction map in Figure 2). The arrow-heads point to the AnTat 1.1 ELC.

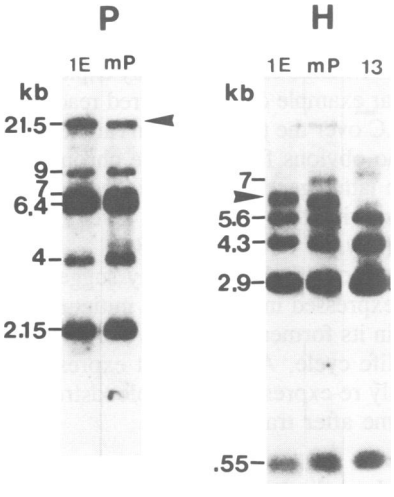

Fig. 4. The AnTat 1.1E ELC is retained after procyclic differentiation in the fly. The figure shows Southern blot analyses of AnTat 1.1 genes in AnTat 1.1E clone (first track in both panels) and in procyclics collected from the midgut of AnTat 1.1E infected flies (second track in both panels). Nuclear DNAs were digested with *Pst*I (left panel) or *Hind*III (right panel) and hybridized as in Figure 3. The third track in the right panel contains AnTat 1.13 DNA, as a non-expressor control. The arrow-heads point to the AnTat 1.1 ELC.

cyclic trypomastigotes collected from the midgut of tsetse flies. These were infected with AnTat 1.1E. Genomic blots revealed all the characteristic fragments of the AnTat 1.1E ELC and its environment, as for instance in Figure 4 which shows the 21.5-kb *Pst*I fragment mentioned above.

Taken together, these results strongly indicate that the 1.1E ELC has been conserved through cyclic transmission and inherited by ensuing bloodstream variants.

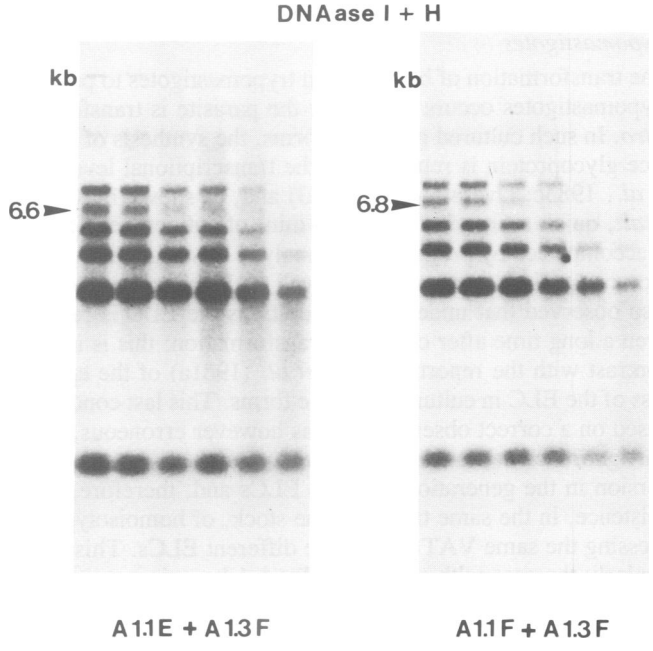

Fig. 5. The AnTat 1.1E ex-ELC is reactivated in AnTat 1.1F. The two blots show the kinetics of DNaseI digestion of AnTat 1.1 specific sequences in AnTat 1.1E (left panel) and AnTat 1.1F (right panel) nuclei. AnTat 1.3F nuclei (to be described elsewhere) were added in both cases in order to provide an inactive DNA reference of adequate target size (the upper band). The test was performed as described previously (Pays *et al.*, 1981) for 0, 2, 4, 8, 16 and 30 min from left to right of each block. Nuclear DNA was digested with *Hind*III and hybridized with a probe made of the 550-bp *Hind*III-*Hind*III fragment of the AnTat 1.1A ELC (see Figure 2). The arrows point to the most DNase I sensitive fragment which is the one containing the AnTat 1.1 ELC.

The same AnTat 1.1 ELC, inactive in the vector, is reactivated without duplication in the 'post-fly' clones AnTat 1.1E and 1.1F

The previous results strongly suggest that the ELC encoding the ingested VAT might be reactivated and transcribed in each corresponding 'post-fly' homoisotype. To substantiate further this hypothesis, we tested the DNaseI sensitivity of the AnTat 1.1 specific sequences in the anTat 1.1E and 1.1F clones. It is known indeed that in isolated nuclei, transcribed genes are preferentially digested by DNase due to an altered chromatin configuration (Weintraub and Groudine, 1976). The results in Figure 5 show that the 6.6-kb and 6.8-kb *Hind*III fragments characteristic of the ELC are digested faster than the other fragments, indicating that the conserved ELC is indeed the variant-specific gene expressed in the AnTat 1.1E and 1.1F clones.

Discussion

In this paper we demonstrate, firstly, that the ELC expressed in the variant ingested by the fly can be retained, although silent, throughout the different developmental stages in the insect vector; secondly, that this ex-ELC can be reactivated, without duplication, to re-express the 'ingested' serotype in bloodstream trypomastigotes of the first wave of parasitemia following transmission. These results are in keeping with the parasitological observations of Hajduk and Vickerman (1981), who reported that the ingested VAT, absent in the metacyclic trypanosome population, is found among the bloodstream forms soon after cyclic transmission.

The ELC is conserved in fly-borne as well as in culture procyclic trypomastigotes

The transformation of bloodstream trypomastigotes to procyclic trypomastigotes occurs soon after the parasite is transferred *in vitro*. In such cultured procyclic forms, the synthesis of the surface glycoprotein is repressed at the transcriptional level (Pays *et al.*, 1983c; Overath *et al.*, 1983) and, as shown by Overath *et al.*, quick repression ($t_{1/2}$ = 30 min) of antigen gene activity is accompanied by progressive proteolytic dissociation of the surface coat. The same groups, as well as Parsons *et al.* (1984), also observed that under these conditions the ELC is retained, even a long time after complete transformation: this is in sharp contrast with the report by Pays *et al.* (1981a) of the apparent loss of the ELC in culture procyclic forms. This last conclusion, based on a correct observation, was however erroneous, due to our ignorance at that time of the occurrence of segmental conversion in the generation of some ELCs and, therefore, of the existence, in the same trypanosome stock, of homoisotypes expressing the same VAT from quite different ELCs. This is particularly the case with several AnTat 1.1 homoisotypes (AnTat 1.1 A, B, C and D), whose ELCs have been generated from the same BC by different segmental gene conversions (Pays *et al.*, 1983a, 1983b, 1983c). A re-evaluation of these experiments as well as recent results presented here confirm that the ELC of the last expressed serotype is retained inactive in the genome after procyclic differentiation *in vitro*.

In addition, the genomic analysis of procyclic trypomastigotes directly isolated from the fly's midgut allowed us to establish that the ELC is retained during the natural cycle of the parasite in the fly. This ELC is maintained, inactive, in its expression site. Since the procyclic transformation is accompanied by various changes in the metabolic pathways, it is possible that antigen gene inactivation constitutes the first detectable change at the time of trypanosome transformation (Overath *et al.*, 1983). Inactivation of the expressing antigen gene on transformation to the procyclic form may be due to activation of a repressor of transcription or, alternatively, to inactivation of a positive activator required for antigen gene expression.

The ELC is also conserved in the metacyclic form and is eventually reactivated early after cyclic transmission

Our observations of the same AnTat 1.1 ELC being conserved throughout the AnTat 1.1E to 1.1F cyclic transmission and in ensuing VATs as well, indicate that the ELC of the ingested VAT is also retained in the metacyclic form. This is further supported by the presence of the same ELC in AnTat 1.30C, a clone directly derived from a single metacyclic trypomastigote and which still expresses its MVAT.

This ex-ELC conservation is apparently complete, except in the case of the first cyclic transmission (AnTat 1.1D→1.1E), in which the size of the 5′ barren region was found to be increased, whereas this region did not alter in size through the second transmission (AnTat 1.1E→1.1F) and all ensuing switchings. This change is probably due to some undetected antigenic variation that perhaps occurred in the first relapses after AnTat 1.1D transmission, the AnTat 1.1E clone having been isolated rather late after infection. An intermediate variant, whose gene would have been activated by the non-duplicative mechanism could have conserved the AnTat 1.1D ELC. Reactivation of the latter could have been accompanied by a change in the size of the 5′ barren region, although no other example substantiates this hypothesis (Michels *et al.*, 1984; E. Pays, unpublished observations). In contrast, the AnTat 1.1F clone has been isolated a short time (< 10 days) after cyclic transmission, making the existence of an intermediate bloodstream variant between AnTat 1.1E and 1.1F unlikely.

Surface antigen genes seem to be expressed by the same mechanisms (duplicative and non-duplicative) in metacyclic and bloodstream trypanosomes (P. Paindavoine, unpublished results). However, it is established that only some genes in the whole collection, those encoding the so-called metacyclic serotypes or MVATs, may be transcribed in the metacyclic form. We therefore suppose that these MVAT genes could have access to a separate control mechanism operating in the tsetse fly. This is supported by the fact that the AnTat 1.1 serotype, when ingested by the fly, is not detected in the metacyclic population, although, as shown here, the AnTat 1.1 ELC is conserved in an apparently unchanged environment, but reappears early after cyclic transmission. In the metacyclic form, such an inactive ELC might be conserved in a 'bloodstream expression site' which could keep the memory of its previous activity state, and be easily reactivable when the trypanosomes again meet 'bloodstream' conditions.

The preferential reactivation of an ELC conserved through cyclic transmission is reminiscent of what happened to some ELCs during antigenic switching in syringe passaged clones of the bloodstream form: indeed, it has been observed, on several occasions, that an ELC is retained inactive after use if the antigen gene expressed in the ensuing variant is activated non-duplicatively (Pays *et al.*, 1983b; Laurent *et al.*, 1984a). In such cases, the ex-ELC appears to be preferentially reactivable, by either the duplicative or non-duplicative mechanisms (Pays *et al.*, 1983b; Laurent *et al.*, 1984b; Michels *et al.*, 1984). This property of a silent ex-ELC cannot be easily explained. For instance, in the particular example of the preferred reactivation of the AnTat 1.3 ex-ELC over the telomeric AnTat 1.3 BC, in the AnTat 1.3B clone, no obvious feature in the chromatin structure differentiates the latter gene from the former, as revealed by the DNase I sensitivity test, or by the level of telomeric DNA modification (Pays *et al.*, 1983b,1984).

In conclusion, our findings strongly suggest that the surface antigen gene expressed in the variant initiating cyclic transmission remains in its former expression site throughout the insect phase of the life cycle. Although not expressed in that phase, it can be readily re-expressed by the bloodstream forms appearing a short time after transmission.

Materials and methods

Trypanosomes

Derivation of trypanosomes from the stabilite EATRO 1125 of *T. brucei brucei* have been described by Van Meirvenne *et al.* (1975). The variants AnTat 1.1D, 1.1E, 1.30C, 1.1F, 1.9B, 1.13 (see pedigree in Figure 1) were cloned after immunological selection and proven by immunofluorescence to be at least 99% homogeneous.

The metacyclic variant AnTat 1.30C was cloned from the first bloodstream trypanosome population arising in a mouse after infection by metacyclic forms. These were obtained by allowing infected tsetse flies to salivate into fresh guinea-pig serum, *in vitro* at 37°C. About 15% of the metacyclic trypanosomes in the flies' saliva turned out to bear the serotype AnTat 1.30, as judged by the immunofluorescence test. The AnTat 1.1F and 1.9B clones were isolated from the population arising, in a similar infection, immediately after the appearance of the major MVATs.

In vitro procyclic forms were derived from the AnTat 1.1E clone and grown in culture as described (LeRay, 1975). Nomenclature of variable antigen types follows the rules recommended by Lumsden (1982).

Cyclical transmission

The AnTat 1.1D and AnTat 1.1E were cyclically transmitted through *Glossina morsitans* (LeRay *et al.*, 1977). *In vivo* procyclics were isolated from the midgut of tsetse flies infected by the AnTat 1.1E variant.

DNA analyses

The procedure for DNA isolation from bloodstream trypanosomes and from culture procyclic forms has been described previously (Pays *et al.*, 1980).

DNA from midgut procyclics has been prepared as follows: ~ 10^6 cells were suspended in 1.2 ml of 100 mM NaCl/250 mM EDTA/10 mM Tris-HCl (pH 8) and lysed by addition of 60 μl 20% SDS. The lysate was treated with 100 μg/ml DNase-free RNase A for 1 h at 37°C and incubated with 100 μg/ml proteinase K for 4 h at 37°C. The DNA was extracted twice by phenol/chloroform (1/1), then with chloroform and ultimately by ether. After dialysis against TE (10 mM Tris-HCl/0.1 mM EDTA, pH 8), for 48 h at 4°C with two changes of the buffer, the DNA was precipitated in ethanol (two volumes) at −70°C.

Southern blot analysis of genomic DNA, using specific nick-translated probes, was performed as described (Southern, 1975; Rigby *et al.*, 1977). The AnTat 1.1 cDNA probe was described by Pays *et al.*, (1980).

Acknowledgements

We thank Dr. Noel B. Murphy for valuable discussion and critical reading of the manuscript. This investigation received support from the Commission of the European Communities (C.E.E., TDS-M-023-B), from the Fonds de la Recherche Scientifique Médicale (F.R.S.M.) and from the ILRAD/Belgian Research Centres Agreement for Collaborative Research (Nairobi). M-F Delauw is a Fellow of the "Institut pour l'Encouragement de la Recherche Scientifique dans l'Industrie et l'Agriculture" (I.R.S.I.A.).

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Received on 10 December 1984