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The control of variant surface antigen synthesis in trypanosomes

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The African trypanosomes belonging to the sub-genus *Trypanozoon* rank among the most versatile and successful protozoan parasites of man and his domestic animals. The species *Trypanosoma brucei* and especially the strains of this species that are non-infectious to man, have become favourites of biochemists in the last two decades. Notwithstanding its complex life cycle in which the trypanosome cycles between its insect vector – the tsetse fly – and mammalian hosts, *T. brucei* is relatively easy to grow in the laboratory. The mammalian bloodstream forms can be reproducibly grown in rats, one rat yielding about 1 g wet weight of trypanosomes in 4 days, while the insect form can be grown in axenic culture.

Three unusual characteristics have endeared the trypanosome to biochemists: the kinetoplast, the glycosome and the phenomenon of antigenic variation (see Fig. 1). The kinetoplast is the specialized part of the mitochondrion that has given its name to the order of Kinetoplastida, to which the African trypanosomes but also the South-American *Trypanosoma cruzi* and the *Leishmanias* belong (see [2]). Fig. 2 shows that the kinetoplast stains with DAPI, a fluorescent dye (4,6-diamino-2-phenylindole) that binds specifically to DNA. In fact, kinetoplast DNA (kDNA) was the first mitochondrial DNA discovered and it is the only mitochondrial DNA readily visible under the light microscope. The reason is that this mtDNA consists of a large network of catenated duplex DNA circles. These circles are of two types: mini-circles and maxi-circles. The maxi-circles have all the properties of a typical mtDNA, but it has been difficult to prove that they actually contain genes required for mitochondrial biogenesis. Only recently has the determination of the complete sequence of the genes coding for the two main RNAs of *T. brucei* mitochondria shown that these are ribosomal RNAs (rRNAs), albeit very small and highly unusual ones [4]. Sequence analysis of additional segments of the maxi-circle by Benne et al. [5] has turned up the gene for apocytochrome *b*, a gene found in all other mitochondrial DNAs thus far. The homology of the *T. brucei* gene with the apocytochrome *b* genes from other eukaryotes is unambiguous but low: maximally 25 % amino acid homology with optimal alignment against 50 % homology for the apocytochrome *b* genes of yeast (another unicellular eukaryote) and man. Obviously, the maxi-circle of *T. brucei* has evolved away from other mtDNAs to the extent that only detailed sequence analysis can detect homology.

The mini-circles, the main component of kDNA, are even more unusual. They are not transcribed and their sequence evolves very rapidly. This has led to a consensus [3, 6] that these mini-circles have a structural rather than a genetic function. Why it should be advantageous to a trypanosome to have its maxi-circles attached to such an enormous and elaborate network of mini-circles has remained a mystery, however.

The second unusual feature of trypanosomes is the presence of glycosomes, microbodies containing most of the enzymes of the glycolytic system. Since their discovery in 1977 [7], glycosomes have been found in all representatives of the Kinetoplastida in which they have been looked for [8]. Moreover, several additional enzymes have been found in these intriguing organelles [9–11] and the recent finding by Opperdoes and co-workers (personal communication) that they also contain a fatty acid β-oxidation system and enzymes of plasmalogen biosynthesis clearly links the glycosome to the peroxisome-glyoxisome family (see [12, 13]). The members of this family perform the most diverse functions in different

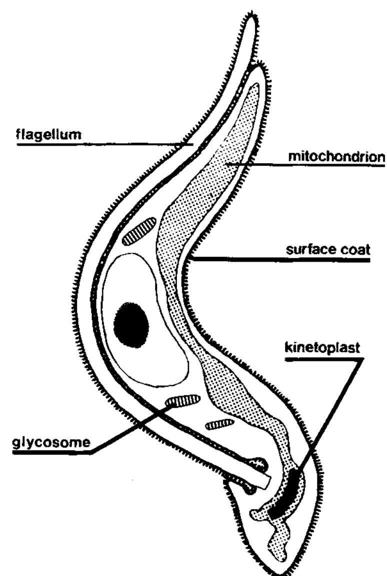

Fig. 1. *Simplified diagram of a cross-section of a trypanosome, showing some of the characteristics that are of major interest to biochemists.* After [1]

Abbreviations. AnTat, Antwerp Trypanozoon antigen type; BC, basic copy; bp, base pair(s); cDNA, complementary DNA; DAPI, 4,6-diamino-2-phenylindole; ELC, expression-linked copy; kb, kilobase pair(s); kDNA, kinetoplast DNA; mRNA, messenger RNA; rRNA, ribosomal RNA; VSG, variant surface glycoprotein.

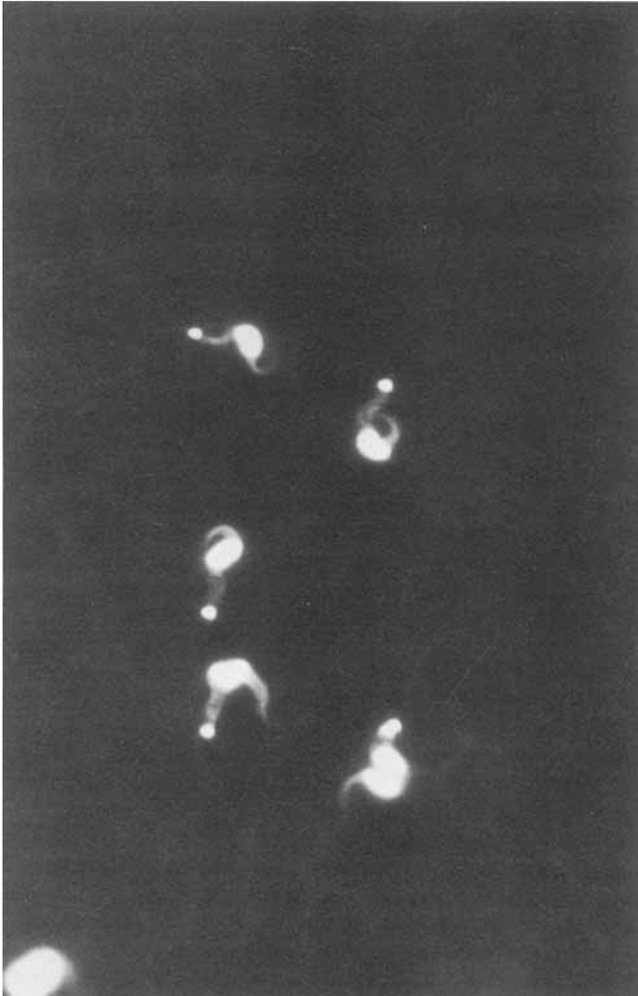

Fig. 2. T. brucei *stained with the DNA-specific fluorescent dye DAPI and visualized by fluorescence microscopy.* The preparations were deliberately over-stained to show the outline of the trypanosome by non-specific dye binding. The terminal bright dot represents the kinetoplast. From [3]

organisms, but the segregation of glycolysis within a microbody seems to be a specific invention of the kinetoplastid flagellates.

The third unusual biochemical feature of African trypanosomes is their ability to evade the host immunological defense by antigenic variation. This aspect of trypanosome biochemistry will be summarized in this lecture.

Antigenic variation: basic biochemistry and early studies on gene rearrangements (reviewed in [1, 14–18])

Let us start out with a brief summary of early studies of antigenic variation. A single trypanosome is able to produce more than 100 different surface coats that have no exposed antigenic determinants in common. In 1975, George Cross demonstrated that this surface coat consists mainly of a single protein species, called by him the 'variant surface glycoprotein' or VSG [19]. From additional studies he concluded that a single layer of VSG molecules covers the entire trypanosome; that the N-terminal domain of this protein is exposed to host antibodies; and that different VSGs differ in the amino acid sequence of this N-terminal domain. These results opened the

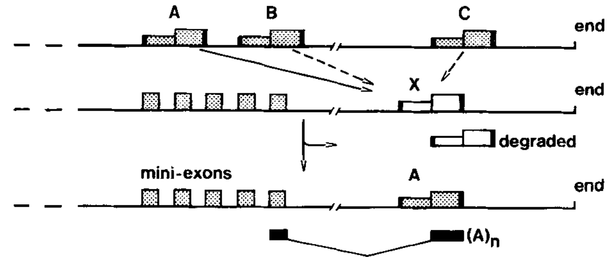

Fig. 3. *Scheme illustrating the expression of VSG genes by duplication of a basic copy gene.* Some trypanosome VSG genes are activated by a duplicative transposition to an expression site that lies at the end of a chromosome. The incoming gene A displaces gene X from the expression site, possibly by a gene conversion process that utilizes short blocks of sequence homology at the edges of the transposed segment (black bars) for alignment. The displaced gene X is degraded. The mini-exons in the expression site provide the 5′ 35 nucleotides of the mature mRNA. Presumably this occurs by splicing of long precursor RNAs as indicated, but this remains to be verified. The mini-exons are present in long tandem arrays of a 1.35-kb repeat, as schematically indicated, but whether all these mini-exons are adjacent to functional transcription starts is not certain. From [20]

way for molecular biologists to tackle the problem at the DNA level. DNA copies of the messenger RNAs (mRNAs) for VSGs were cloned as recombinant DNA in *Escherichia coli* and these complementary DNA (cDNA) clones were then used to demonstrate three main points.

1. In contrast to immunoglobulin genes, VSG genes are not assembled from gene segments. Each VSG is encoded by a separate gene and molecular hybridization studies have indicated that there are some 10^3 potential VSG genes per trypanosome nucleus, occupying about 10% of the genome.

2. A VSG mRNA is only detectable when the corresponding VSG is synthesized. This led to the conclusion that VSG gene expression is controlled at the level of RNA synthesis, processing or stability.

3. Expression of some VSG genes involves the duplication of a basic copy (BC) gene and the transposition of the duplicate to an expression site located at the end of a chromosome, as illustrated in Fig. 3. We refer to this transposed duplicate as the 'expression-linked (extra) copy' or ELC.

The duplicative transposition of VSG genes: the transposition mechanism

In rodents, well-adapted laboratory strains of *T. brucei* switch VSGs at a rate of $10^{-5}-10^{-6}$ per division [21]. This rate is not detectably affected by antibody or other external stimuli and it is too low to allow a direct analysis of DNA molecules that may be involved as intermediates in switching. A further complication has been that ELC genes have resisted cloning in *E. coli.* This has prevented the direct comparison of BC and ELC and encumbered the identification of the exact borders of the transposed DNA segment. To obtain information about the transposition process we have made detailed restriction maps of ELCs in nuclear DNA and compared these with the sequence of the corresponding BC genes and VSG mRNAs. This has led to the transposition-displacement model shown in Fig. 4A. This model is mainly based on three observations.

1. The DNA segment transposed starts about 1.5–2 kb in front of the coding sequence and terminates within or at the end of this sequence. This has now been observed for three

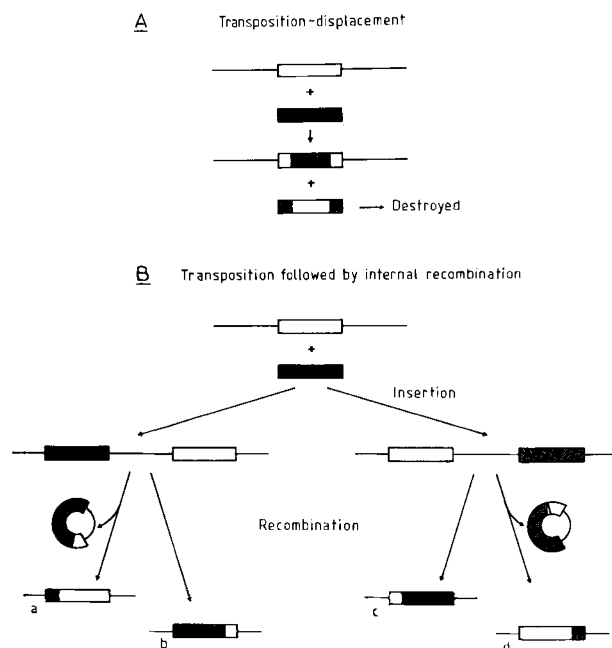

Fig. 4. *Speculative models for the formation of expression-linked copies (ELCs) of VSG genes.* (A) Transposition displacement. (B) Transposition followed by internal recombination. The white block represents the resident ELC, the black block the incoming BC gene that becomes the new ELC. From [17]

different BC genes in the 427 strains of *T. brucei* studied in this laboratory [22] (and unpublished results).

2. The 3′ terminus of a VSG mRNA does not necessarily contain the same nucleotide sequence as the corresponding BC gene [23]. The duplicative transposition may, therefore, lead to replacement of the 3′ end of a VSG gene.

3. The 5′ edge of the transposed segment of the 118 gene contains several imperfect repetitions of a 70-bp sequence [24]. By molecular hybridization these repeats have been found in front of all VSG genes analysed [22]. The exact 3′ edge of the transposed segment varies and lies either before, in or behind the 3′ non-translated part of the mRNA [25]. There are several sequence motifs in this 3′ non-translated region shared by many VSG mRNAs (see [16, 24, 25]). The most striking of these is a 14-mer sub-terminal sequence that is shared by all functional VSG mRNAs [16, 26].

The model in Fig. 4A suggests that the transposition involves a recombination between the incoming gene and the resident ELC, the sequences at the edges of the transposed segment providing the limited homology required to displace the resident ELC by gene conversion. This model predicts that the new VSG mRNA (after switching) may carry the 3′ end of the old VSG mRNA (made before switching). This simple prediction has been difficult to verify for technical reasons. We have found, however, that trypanosome variants that appear in consecutive parasitaemic waves of a chronic infection may have mRNAs with the same 3′ terminus, although the remainder of the mRNA is totally different [25] (and unpublished results). Since there are no two VSG BC genes among the 12 analysed by us or others that have the same 3′ end, it is unlikely that this result can be attributed to the consecutive expression of VSG BC genes that happen to have the same end.

How the duplication-transposition proceeds in detail remains unknown. The analogy with the duplicative transposition resulting in the mating-type switch in yeast seems obvious [27] and has recently been stressed [28]. A basic difference between the trypanosome and yeast transposition, however, is the degree of homology between donor and acceptor sequence. In yeast, the transposed sequence is flanked by homologous regions of several hundred base pairs [29]. At the 3′ edge of the transposed segment in trypanosomes the homology is limited to scattered oligonucleotides. Some BC genes even lack the characteristic 14-mer present at the 3′ terminus of VSG mRNAs [24]. At the 5′ edge the degree of homology is not known, but it cannot be high as it is only detected by hybridization at low stringency. Since the rate of homologous recombination rapidly falls off when the homology is reduced below 100 bp [30], it is doubtful whether trypanosome transposition could involve a gene conversion based on homologous recombination, as employed by yeast.

Although the transposition-displacement (gene conversion) model is the simplest one that accounts for the experimental results, more complex transposition events cannot be excluded. A copy of the BC gene might, for instance, be inserted into the barren regions flanking the expression site, followed by a recombination eliminating one of the genes, as depicted in Fig. 4B. These alternatives are difficult to assess at the low rates of switching displayed by the *T. brucei* strains studied.

How does transposition activate a VSG gene?

The key finding that provided an answer to this question was the demonstration that BC genes are incomplete [23, 31]. Sequence comparison of mRNA and corresponding BC gene showed that the 5′ 35 nucleotides of the mRNA are not encoded contiguously with the remainder of the mRNA sequence in the BC. Since the missing 35-bp mini-exon was not found elsewhere in the transposed segment [24], we have proposed that it is provided by the expression site, as schematically indicated in Fig. 3. We assume in this scheme that the expression site contains one or more mini-exons and that transcription can start 5′ of each of these. The resulting long precursor is processed to yield mature VSG mRNA by appropriate splicing steps. If there is only one functional expression site per trypanosome and if a VSG gene that enters the site displaces the preceding one as is shown in Fig. 4A, the trypanosome would ensure that only one gene is expressed at a time.

Hybridization analysis of trypanosome nuclear DNA has indeed turned up the missing mini-exons [32]. The mini-exon sequence is flanked at its 3′ side by dG-dT and the main exon at its 5′ side by dA-dG. This is in line with the idea that mini-exon and main exon are joined by splicing and that trypanosomes use the polymerase II dG-dT/dA-dG splice borders employed by all other eukaryotes. Unexpectedly, however, there are at least 200 mini-exons per nucleus and these are embedded in tandem repeats of a 1.35-kb DNA sequence. An optimistic interpretation of this result is that most of the mini-exons reside in the expression site and can be used to start synthesis of precursor RNA that is spliced to yield mature VSG mRNA. Such a repetitive promoter might be required to produce sufficient mRNA for the VSG which constitutes 7–10% of the cellular protein.

Thus far all experiments done to verify the other features of the scheme in Fig. 3 have yielded negative results. There is no mini-exon within 10 kb of the transposed segment in the expression site and the longest RNA, that hybridizes either with mini-exon DNA probes or with DNA probes corre-

sponding to the main exon of the VSG gene, is 6 kb. Obviously, there are trivial explanations for these results, but we cannot exclude outlandish alternatives like an independent transcription of mini-exon and main exon from non-contiguous sites in the genome followed by bimolecular splicing to yield mature mRNA.

Intermezzo: chromosome ends grow in trypanosomes

We have mentioned already that the expression site is located at a chromosome end. Since trypanosomes, like other primitive eukaryotes, do not condense their chromosomes during mitosis into cytologically recognizable structures, the telomeric location of the expression site was deduced from biochemical experiments showing a duplex discontinuity downstream of the ELC [33].

In our analysis of ELCs we noted a variation in the size of the telomeric segment downstream of the ELC and attributed this to frequent duplications/deletions in a repetitive DNA sequence [25]. To pin-point these alterations we have followed the telomere for more than 300 trypanosome generations by serially transferring a homogeneous population from mouse to mouse under conditions where VSG switching is not selected for by antibody production. The baroque result obtained was that the telomeric segment increased monotonically in length at a rate of about 10 bp per generation [34]. This is not a peculiarity of the expression site telomere, but a general feature of trypanosome telomeres [34, 35] (and unpublished results). Obviously, there must also be a process that shortens these telomeres or the trypanosome would eventually burst. We have noted that trypanosome telomeres may lose as much as 17 kb under heat stress [34]. What induces shortening under physiological conditions is not known.

A popular model for the replication of chromosome ends involved a terminal DNA hairpin. This model can be easily modified to account for chromosome growth (see [34]). We have recently managed to clone a telomere as recombinant DNA in *E. coli*, however, and found that the most terminal sequence consists of tandem repeats of the hexamer sequence 5′d(C-C-C-T-A-A)3′ (unpublished results). If there is a hairpin, it must be at the very end and absent in our clones. It is possible, therefore, that the replication of trypanosome telomeres does not involve terminal palindromes. These results will be presented in detail elsewhere.

Telomeric gene conversion

Although most of the VSG BC genes occupy a chromosome-internal position, a substantial fraction is located at chromosome ends, like the expression site [16, 36–38]. Such genes can also move into the expression site by a duplicative transposition, but this process differs from the transposition of internal genes in one important aspect, schematically indicated in Fig. 5. Whereas the transposed segment of internal genes always ends within or at the end of the gene, the telomeric genes can be transferred together with a downstream segment. This segment may actually include the entire telomere, since we cannot distinguish the most terminal segment of one telomere from another for lack of distinctive restriction sites. Our evidence for this modified duplicative transposition, dubbed telomeric gene conversion rests on two examples. First, we have recently shown that the telomeric 221 gene in our trypanosome stock can be transferred to the expression site in this fashion (unpublished). The cross-over in front of the genes

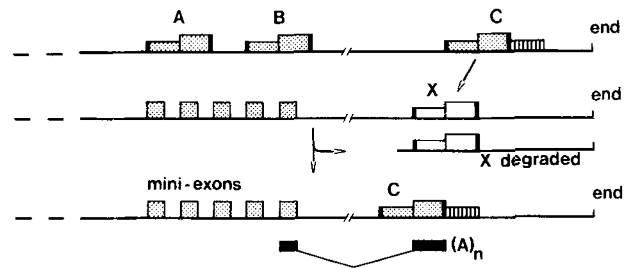

Fig. 5. *Scheme illustrating how some telomeric VSG genes can be activated by a telomeric gene conversion.* See Fig. 3 and text for explanation. From [20]

lies 1–5 kb before the coding region; the transposed segment includes at least 0.8 kb behind the gene, but may extend to the chromosome end. The second example comes from a detailed analysis of an unusual event in one of our trypanosome variants that expresses the 118 gene. Fig. 6 shows a comparison of the ELCs from four independently isolated variants expressing the 118 gene. One of these, the 118c, is unusual in that it has a 0.9-kb segment (called 'TIS3') behind the gene that contains restriction enzyme cleavage sites. We have shown that this segment is not co-transposed with the 118 gene to the expression site, but comes from elsewhere in the genome [25]. Recent (unpublished) experiments have demonstrated that 'elsewhere' is in fact a sub-telomeric location. The TIS3 segment is located downstream of a telomeric VSG gene and we infer that telomeric gene conversion must have brought in TIS3 into the expression site together with the adjacent VSG gene, designated 1.1006. Subsequent transposition of the 118 gene then displaced the 1.1006 gene from the expression site. The cross-over has been precisely located in a region where the 118 gene and the 1.1006 gene have a 16-bp stretch of homology.

Although telomeric gene conversion is clearly a variant of the duplicative transposition observed for chromosome-internal VSG genes, there are sufficient differences between the two processes to entertain the possibility that they are catalysed by different enzymes and start at different positions: the transposition of internal genes from the edge (or edges) of the transposed segment (donor or acceptor); the transposition of telomeric genes from the long regions of homology at the tip of the telomere. A high rate of telomeric gene conversion, not necessarily involving the expression site telomere, can also account for several puzzling observations reported in the literature or made in our laboratory. Before telomeric VSG genes were even shown to be telomeric, their unusual instability was already noted. Williams and co-workers demonstrated that these genes may change in copy number during a chronic infection, genes coming and going without apparent connection with expression [39–41]. We showed that the telomeric 221 gene is absent in 12 out of 13 trypanosome strains [42] and, more recently, that it may also be easily lost in our 427 strain. Analogous results were obtained with two other telomeric genes (unpublished). Another example is the partial replacement of the AnTat 1.1 ELC gene in variant AnTat 1.1B observed by Pays and co-workers [43, 44]. This result can now be readily explained by telomeric gene conversion that has gone 600 bp into the gene before stopping [35].

If there are many telomeric VSG genes (see below), if internal genes can transpose to many telomeres, and if partial telomeric gene conversion is a frequent event, each trypanosome would be able to make its own cocktail of (telomeric)

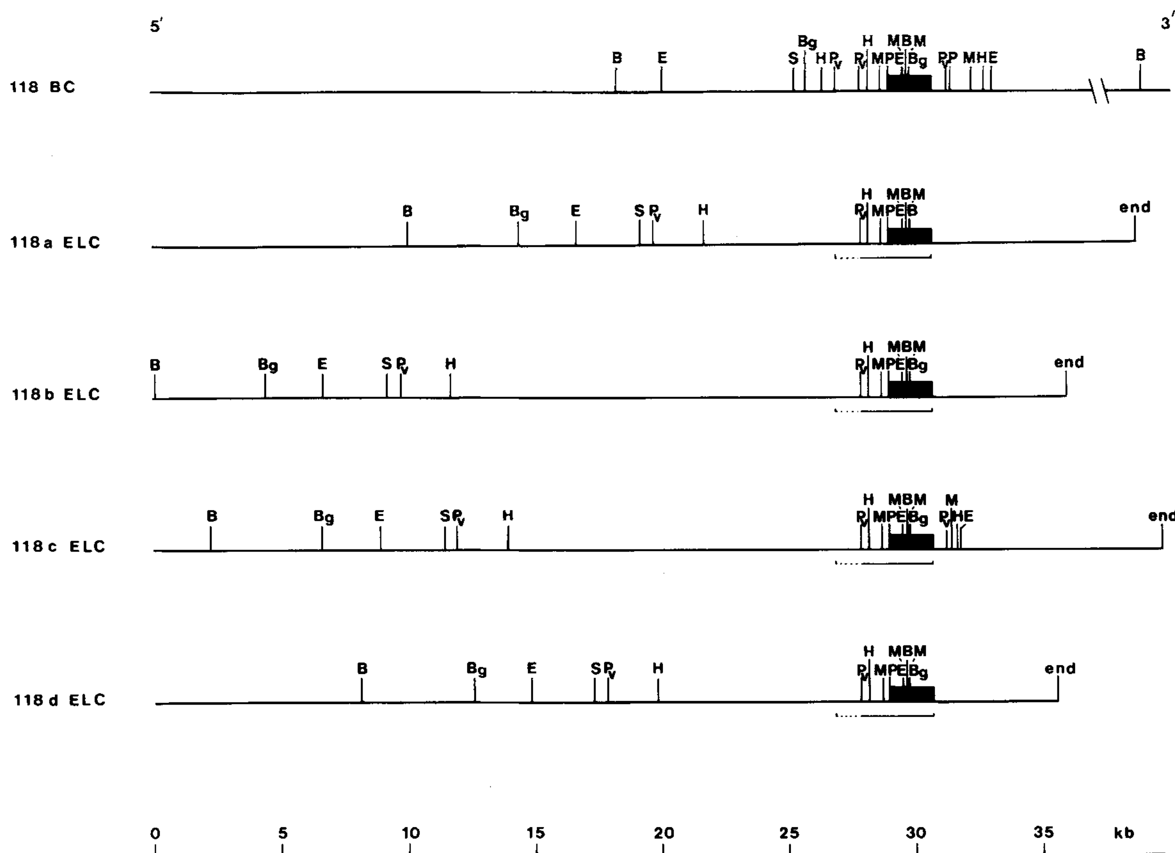

Fig. 6. *Physical maps of the VSG 118 basic copy (BC) gene and the ELC genes from four independently arisen trypanosome clones that make VSG 118.* The main exon of the 118 gene is indicated with a box, the 35-bp mini-exon donated by the expression site (see text) has not yet been localized precisely, but must lie 5' of the upstream *Eco*RI (E) site. The lines beneath the ELCs indicate the segment copied from the BC. 'end' indicates a chromosome end (see text). Abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I; St, *Stu*I; X, *Xba*I. From [25]

VSG genes. This could speed up the evolution of antigenic repertoires as discussed in more detail in previous papers [16, 17, 35, 38, 42] and by Pays et al. [45].

Some telomeric VSG genes can be activated without detectable duplication

The initial analysis of VSG genes in 1979–1980 already showed that some VSG genes are activated without the concomitant appearance of an extra ELC. The 221 gene in our *T. brucei* strain [16] and several genes studied by Williams and co-workers [39, 40] belong to this class. We now know that genes of this class are invariably located at telomeres [16, 17, 35–38] and that at least one representative of this class, the 221 gene, can also be activated by telomeric gene conversion. We also know that trypanosomes contain some 100 mini-chromosomes. Since mini-chromosomes are absent in an insect trypanosome species, *Crithidia fasciculata*, that can not undergo antigenic variation, it looks as if the mini-chromosomes have evolved specifically to allow an increase in the number of chromosome ends available for VSG genes. Telomeric VSG genes should, therefore, be important to *T. brucei* and, hence, to detailed understanding of the mechanism of antigenic variation.

How could a telomeric gene be activated without duplication? There are many telomeric VSG genes and, in addition, there is a telomeric expression site into which chromosome-internal genes move by duplicative transposition and telomeric genes by telomeric gene conversion. How can one have a non-duplicative activation of VSG genes in such a complex system without expressing more than one VSG gene at a time? To solve this conceptual problem we proposed [18] the chromosome-end exchange model illustrated in Fig. 7. This model postulates that telomeric genes can move into the expression site by a reversible and reciprocal telomere translocation. The exchange of position between telomeric gene and ELC simultaneously activates transcription of the telomeric gene and shuts off transcription of the ELC. Several predictions of this model have now been verified.

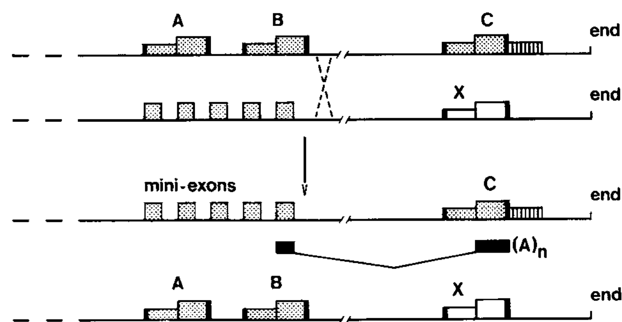

Fig. 7. *Speculative scheme showing how telomeric VSG genes could be activated by a reciprocal translocation.* See Fig. 3 and text for explanation. From [20]

1. When the 221 gene is switched on without detectable duplication it produces an mRNA carrying the same 35-nucleotide 'mini-exon' sequence at its 5′ end as the chromosome-internal genes that procure this sequence by duplicative transposition into the expression site [46]. The 35-nucleotide mini-exon is not present within 8 kb of the main exon of the 221 gene [18].

2. Expression of VSG genes can be switched off without loss of the ECL involved in expression. This was first observed for the 121 gene in our strain [27] and it has been studied in more detail with the 118 gene [35] (and unpublished results). We have shown, for instance, that variant 118b can switch to expression of VSG gene 1.8 without loss of the 118b ELC. This 1.8 variant gives rise to 118 expressors that produce a 118 VSG mRNA with the same 3′ end as that in variant 118b. Moreover, the size of the 5′ ELC fragments in these new 118b′ variants is the same as in 118b. Since there are (on the average) three copies of the 1.8 gene in our strain, two of which telomeric, these results are fully in agreement with the predictions of the model in Fig. 7.

Obviously, however, the same results would be obtained if the 221 gene were activated *in situ* and if activation involved a promotor-mini-exon area more than 8 kb upstream of the gene. There is only one critical prediction of the model in Fig. 7 and that is exchange of genes between telomeres. We have tested this prediction by two types of experiments, both negative thus far. First, we have tested by blot analysis of nuclear DNA whether we can detect a change in upstream restriction sites of the 221 gene and the ELC when the 221 gene is switched on and the ELC is switched off. No change was detected as far upstream as we can look with the probes available (about 50 kb). Second, we have analysed the putative chromosome translocation more directly using a novel gel system that allows the fractionation of chromosome-sized DNA molecules [47]. No transfer of the 221 gene to the expression site chromosome was found when this gene was switched on without duplication, whereas this transfer was readily detected when the 221 gene was activated by duplicative transfer to the expression site by means of telomeric gene conversion [38] (and inpublished results). In these recent experiments we have also found that only part of the mini-exons are located on the expression site chromosome.

Although these results do not exclude the possibility that telomeric genes can be activated by a reciprocal translocation with the expression site chromosome, it now seems likely that there must be another way of activating and inactivating VSG genes than the three pathways depicted in Figs. 3, 5, and 7. To explain how the trypanosome manages to express one gene at a time, three alternatives can be considered.

a) There is a single mobile activator element required for activation of genes at telomeres. Switching occurs either by moving a gene into the telomere that contains the element or by moving the element to another telomere.

b) There are several telomeres that can act as an expression site and cross-talk between these telomeres limits expression to one of them. This possibility has also been raised by others [40, 48], but it is not easy to translate this idea into a detailed model (cf. [17]) and no such model has been brought forward.

c) There are several telomeres that can act as an expression site and activation and inactivation of these telomeres occurs randomly and at low frequency. Intuitively, this seems a wasteful and clumsy way of regulating VSG gene expression, but we have calculated that this waste is negligible in a chronic infection, which is inherently wasteful anyhow in terms of trypanosome lives. The possible molecular basis of such a random telomere activation/inactivation process will be considered elsewhere.

Outlook

Although we still do not understand how antigenic variation works in detail, there is little reason for complaint. The 4 years that have elapsed since the first cDNA clones of VSG mRNAs were isolated, have yielded a host of interesting biochemical results. The duplication-transposition control of VSG gene expression is one of the most intricate systems for the control of gene expression yet discovered, only paralleled by the combinatorial diversity generated during the assembly of antibody genes. The baroque behavior of trypanosome chromosome ends is an added bonus and this is obviously not the end of the story. The high advantage to trypanosomes of generating new VSG genes readily allows the study of evolutionary mechanisms that only rarely surface in other organisms. The presence of some 100 mini-chromosomes [36, 38, 49] that seem to have arisen in response to the need for more VSG genes at chromosome ends, provides readily available material for the analysis of intact interphase chromosomes. Thus far the trypanosome has not disappointed the biochemists that have chosen it as pet organism and we are confident that there are more surprises in store.

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