Cell, Vol. 95, 839–846, December 11, 1998, Copyright ©1998 by Cell Press

A *VSG* Expression Site–Associated Gene Confers Resistance to Human Serum in *Trypanosoma rhodesiense*

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Summary

Infectivity of *Trypanosoma brucei rhodesiense* to humans is due to its resistance to a lytic factor present in human serum. In the ETat 1 strain this character was associated with antigenic variation, since expression of the ETat 1.10 variant surface glycoprotein was required to generate resistant (R) clones. In addition, in this strain transcription of a gene termed *SRA* was detected in R clones only. We show that the *ETat 1.10* expression site is the one selectively transcribed in R variants. This expression site contains *SRA* as an expression site–associated gene (*ESAG*) and is characterized by the deletion of several *ESAG*s. Transfection of *SRA* into *T.b. brucei* was sufficient to confer resistance to human serum, identifying this gene as one of those responsible for *T.b. rhodesiense* adaptation to humans.

Introduction

Trypanosoma brucei is the paradigmatic species of African trypanosomes, protozoan flagellate parasites transmitted by tsetse flies. These organisms are particularly well studied for their spectacular mechanism of antigenic variation, a process by which the major surface antigen, the variant surface glycoprotein (VSG), is continuously changed to escape the immune defenses of the mammalian host (Cross, 1978). *T. brucei* consists of three subspecies that are indistinguishable by conventional morphological, biochemical, and antigenic criteria but differ by their geographical distribution and host specificity. *T.b. brucei* causes "nagana" in cattle but is not pathogenic in humans because this subspecies is lyzed by haptoglobin-related proteins associated with a subfraction of high-density lipoproteins (HDL) in human serum (reviewed in Tomlinson and Raper, 1998). *T.b. rhodesiense* and *T.b. gambiense* are resistant to normal human serum (NHS), enabling them to cause "sleeping sickness" in humans. The mechanism of resistance to NHS has yet to be established, although it is linked to a defect in the uptake of the lytic factor (Hager and Hajduk, 1997).

In *T.b. rhodesiense* resistance to NHS is reversible and relatively unstable. Cloned populations of *T.b. rhodesiense* exist in both forms that are sensitive (S) or resistant (R) to NHS. Interestingly, in the Edinburgh trypanozoon antigen type (ETat 1), strain resistance was found to be linked to the expression of a given VSG, termed ETat 1.10 (Van Meirvenne et al., 1976). This VSG was always the first to be detected when cloning R variants, and successful cloning of new R forms was only achieved when ETat 1.10 was eliminated by homologous antisera. Moreover, ETat 1.10 has never been obtained in the S form. Despite the requirement of ETat 1.10 for resistance, the VSG per se was not important, since clones derived from ETat 1.10 and expressing other VSGs, for instance ETat 1.2, were found in both R and S forms (Van Meirvenne et al., 1976). Thus, while in the ETat 1 strain resistance is linked to antigenic variation it does not depend on the VSG. This phenomenon may be related to the structure of the transcription unit of the *VSG*. This unit, located in a telomeric expression site (ES), is polycistronic and contains several genes, termed *ESAG*s for expression site–associated genes, in addition to the *VSG* (Cully et al., 1985; Kooter et al., 1987; Pays et al., 1989). Approximately 20 different ESs can be used alternatively for VSG expression (Navarro and Cross, 1996). Antigenic variation occurs through transcriptional switching between ESs, a process termed in situ (in)activation, or gene conversion targeted to the *VSG* (for recent reviews, see Borst et al., 1998; Cross et al., 1998; Pays and Nolan, 1998). These properties provide a possible explanation for the antigenic variation-linked resistance to NHS in *T.b. rhodesiense*, in which the gene responsible for resistance would be an *ESAG* only present in some ESs, namely the *ETat 1.10* ES in the ETat 1 strain. Switching to this site would confer resistance, which could be conserved in clones derived from ETat 1.10 provided antigenic variation does not result from a switch of ES but is performed by gene conversion only replacing the *VSG* gene by another in the same ES.

In the ETat 1 strain, differential cDNA screening between S and R clones identified a transcript present only in R forms (De Greef et al., 1989). The gene for this transcript, termed *SRA* for serum resistance-associated, was found to encode a VSG-like protein (De Greef and Hamers, 1994). In this study, we show that *SRA* is an *ESAG* of the *ETat 1.10* ES, in agreement with the hypothesis presented above. Moreover, *SRA* was found to confer resistance to NHS when transfected into *T.b.*

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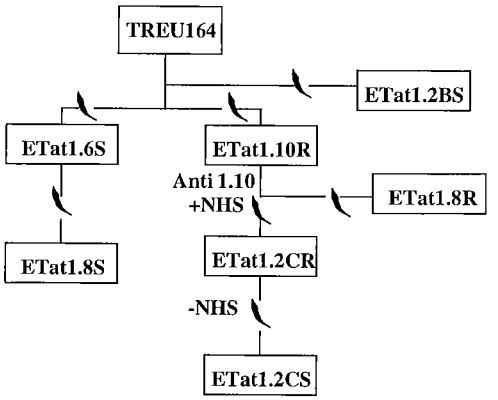

Figure 1. Pedigree of the *T.b. rhodesiense* ETat 1 R and S Clones

TREU164 is a stabilate obtained after 21 passages in mice and rats, from a tsetse fly captured in Uganda in 1960. The parasite symbol depicts the cloning of trypanosomes, and NHS indicates that a treatment with human serum was performed before and during injection into mice. In order to select antigenically distinct R clones from ETat 1.10R, selective elimination of ETat 1.10 was accomplished by lytic neutralization with homologous antiserum.

brucei. Therefore, the antigenic variation-linked expression of *SRA* appears to underly resistance to human serum in at least some strains of *T.b. rhodesiense*.

Results

Antigenic Variation-Linked Switching from Sensitivity to Resistance in ETat 1

In the ETat 1 strain of *T.b. rhodesiense*, the selection of R clones systematically resulted in the expression of the ETat 1.10 VSG, and counter-selection was necessary in order to generate R clones expressing different VSGs (Van Meirvenne et al., 1976). The genetic events underlying these phenomena were studied in cloned variants whose pedigree is shown in Figure 1. An ETat 1.2S clone was derived from the initial stabilate (2BS) and compared with two other clones expressing the same VSG, respectively, in R form derived from ETat 1.10R and in S form derived from the R form (2CR and 2CS). Additional clones in R and S forms were included in this study, such as ETat 1.8R and 1.8S, both of which were obtained independently (Figure 1). Figures 2–4 depict the genetic characteristics of the series 2BS-10R-2CR-2CS followed by 8R and 8S, in this order.

Mechanisms of Antigenic Variation in the ETat 1.10R-2CR-2CS Clone Derivation

The *ETat 1.10R*, *1.2CR*, and *1.2CS VSG* cDNAs were cloned to generate hybridization probes. The *ETat 1.2CR* and *1.2CS* cDNAs were found to be identical. In the ETat 1.2BS clone, the *ETat 1.10* probe hybridized with three DNA fragments (Figure 2A, first lane). In ETat 1.10R, despite the antigenic variation event leading to expression of *ETat 1.10*, no difference was observed in this pattern (second lane). The absence of DNA rearrangement suggested that *ETat 1.10* was activated in situ, through ES switching. The active *ETat 1.10* gene was contained in a 6 kb EcoRI fragment, since this fragment was hypersensitive to DNAaseI (Figure 2A, arrowhead). Interestingly, this gene was lost in ETat 1.2CR, a clone

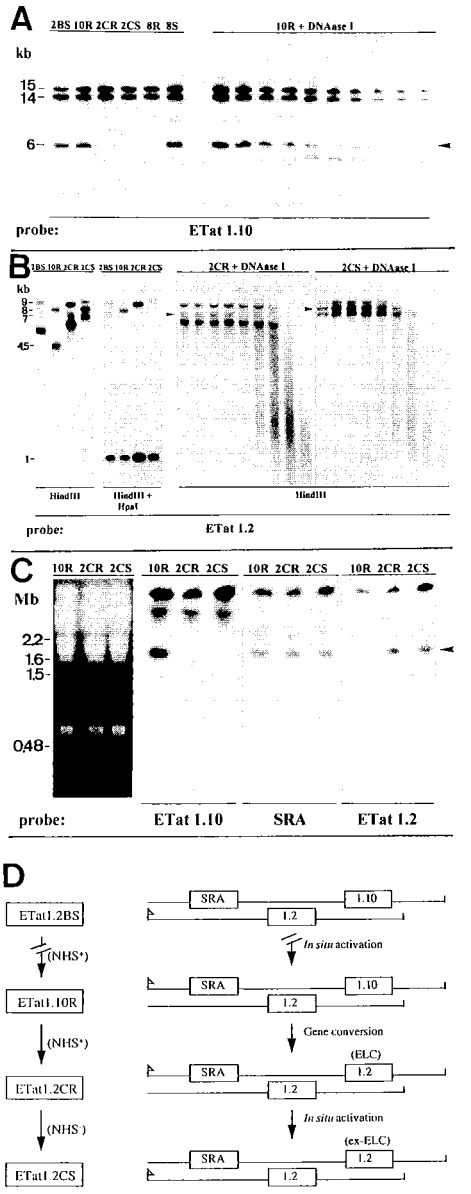

Figure 2. Genetic Events Underlying Antigenic Variation

(A–C) Results of Southern blot hybridization with the indicated probes, whose size is represented by thick bars under the maps in Figure 4B. In (A), the genomic DNAs were digested with EcoRI. Where indicated, increasing amounts of pancreatic DNAase I were added to isolated nuclei prior to DNA extraction (Pays et al., 1981). (C) Results of chromosomal PFGE analysis, an ethidium bromide staining of the gel being shown at the left. (D) summarizes the results. In the ES maps, the flag represents the active transcription promoter, the boxes represent genes, and the terminal vertical bar depicts the chromosome end.

directly derived from ETat 1.10R (third lane of left panel), suggesting that gene conversion associated with antigenic variation led to the replacement of this gene by the copy (ELC, for expression-linked copy) of *ETat 1.2*. The loss of *ETat 1.10* was also observed in Etat 1.8R (fifth lane) and in other R clones obtained independently by neutralization of ETat 1.10 with specific antibodies (data not shown).

In the ETat 1.2BS clone *ETat 1.2* was present as two

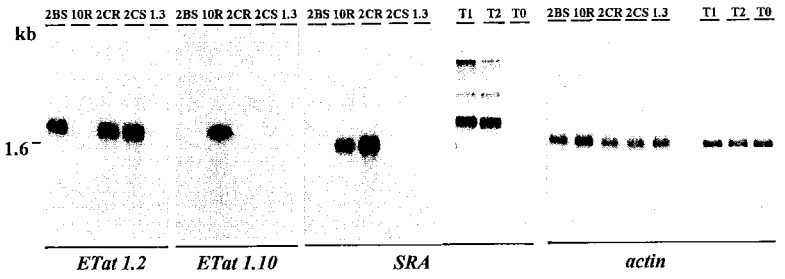

Figure 3. R-Linked Transcription of the *SRA* Gene

The indicated probes were hybridized with Northern blots of total RNA (4 μg) from the different *T.b. rhodesiense* clones (2BS, 10R, 2CR, and 2CS) and from *T.b. brucei* AnTat 1.3A (1.3), as well as with *T.b. brucei* transformants (T1 and T2, two independent *SRA* transformants; T0, control transformant). Exposure time was 1 hr, 8 hr, and 4 days for the *VSGs*, *SRA*, and *actin* probes, respectively, except in the T1, T2, and T0 *SRA* lanes where exposure was for 20 hr. The significance of the two upper *SRA* transcripts in T1 and T2 is unclear, although the middle one may correspond to dicistronic *SRA-HygroR* mRNA.

HindIII fragments (Figure 2B, left panel, first lane). In ETat 1.10R (second lane) the size of these fragments was altered, indicating a telomeric location, which was confirmed by genomic mapping (data not shown). A HindIII + HpaI digest revealed the presence of a HpaI site downstream from the gene in one of the two copies (bottom band in second panel). Expression of the *ETat 1.2* gene in ETat 1.2CR was linked to the appearance of a third HindIII fragment, which migrated as a smear (Figure 2B, third lane of first panel and third panel, arrowhead) but was more clearly evidenced by the doubling of the hybridization intensity of the 1 kb HindIII-HpaI fragment. This smear is typical of actively transcribed telomeric DNA (Pays et al., 1983), suggesting that the additional fragment contains the active *ETat 1.2* ELC. This was confirmed by the DNAase I sensitivity assay, which showed that this fragment was more sensitive than the two others (third panel, arrowhead). Thus, in the ETat 1.2CR clone, activation of *ETat 1.2* was due to gene conversion. According to this interpretation, in 2CR the *ETat 1.2* ELC replaced *ETat 1.10*. This was verified by chromosomal DNA analysis. The *ETat 1.10* probe hybridized to several chromosomes, one of which (approximately 1.6 Mb) contained the copy lost in 2CR and thus harbored the active *ETat 1.10* ES (Figure 2C). In clone 2CR the *ETat 1.2* ELC mapped to the same chromosome, since the intensity of labeling was increased 2-fold in this band in 2CR compared to 10R (Figure 2C, arrowhead). Therefore, in 2CR the *ETat 1.2* ELC replaced the active *ETat 1.10* gene in the same ES. Finally, in clone ETat 1.2CS the additional *ETat 1.2* fragment was no longer diffuse (Figure 2B, fourth lane of first panel), suggesting transcriptional inactivation, which was confirmed using DNAase I (fourth panel, arrowhead). In this clone the upper band became the most sensitive to DNAase I, indicating that the switch from 2CR to 2CS was due to in situ activation.

These data are summarized in Figure 2D. In ETat 1.10R, in situ activation of the *ETat 1.10* ES occurred. In ETat 1.2CR, VSG switching in the presence of NHS was due to gene conversion resulting in the replacement of *ETat 1.10* by the ELC synthesized on one of the two telomeric *ETat 1.2* copies. In the absence of NHS, in situ activation allowed the reexpression of the *ETat 1.2* ES already functional in the 2BS clone. As a consequence, the previously active *ETat 1.2* ELC was conserved but silent (ex-ELC) in the *ETat 1.10* ES. These conclusions were confirmed by RT-PCR using pairs of primers specific to *ESAG 7/6*, which allowed the discrimination between the different ES transcripts (data not shown). Overall these results suggested that activation of the *ETat 1.10* ES is required to generate R clones and that in the absence of NHS the *ETat 1.10* ES is counterselected.

In These Clones, Transcription of *SRA* Is Associated with Resistance to Human Serum

In accordance with previous results (De Greef et al., 1989), *SRA* transcripts were only detected in R and not in S clones (Figure 3). The detection of *SRA* transcripts in 2CR but not in 2CS was especially significant, since the latter clone was directly derived from the former and expressed the same VSG. The level of *SRA* mRNA was approximately one-eighth that of the *VSG* mRNA, both being much more abundant (30- and 250-fold, respectively) than transcripts from housekeeping genes such as *actin*.

The *SRA* Gene Is Contained in the *ETat 1.10* ES

As shown in Figure 4A, the *SRA* probe hybridized with several genomic fragments, the largest of which exhibited the characteristics of telomeric DNA, including extensive terminal size variation (arrows and arrowheads in second panel). Significantly, this fragment also appeared to hybridize with the *ETat 1.10* probe except in variants where the active *ETat 1.10* gene was lost following gene conversion (first panel, arrows). These observations strongly suggested that the telomeric *SRA* fragment contained the *ETat 1.10* ES. Moreover, in the 2CR and 2CS clones where the *ETat 1.10* gene was lost, the telomeric *SRA* fragment (19 kb) hybridized with the *ETat 1.2* probe and clearly this fragment contained the *ETat 1.2* ELC (third panel, arrowheads). DNAase I sensitivity analysis also supported the colocalization of *SRA* and *ETat 1.2* ELC within the very same fragment (data not shown). Therefore, an *SRA* copy is linked to the active *VSG* in R clones only. This conclusion was in agreement with the colocalization in a 1.6 Mb chromosome of *SRA* and both *VSGs* expressed in R clones (Figure 2C).

The restriction maps of the *ETat 1.10R* and *ETat 1.2CR* ESs are presented in Figure 4B. Two large regions, immediately upstream and downstream from the *VSGs*, appeared to be devoid of restriction sites. These regions probably contain arrays of 76 bp repeats ("barren" region) and telomeric repeats, respectively (Bernards et

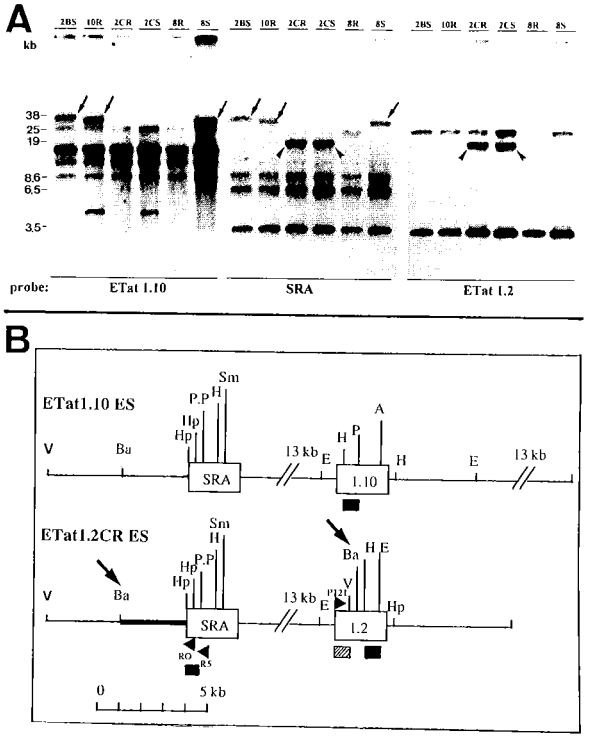

Figure 4. Localization of an *SRA* Copy in *R-VSG* ESs

(A) Results of hybridization with BamHI + EcoRV digests of genomic DNAs.

(B) Restriction maps of the *VSG* ESs in the two R clones, as determined from Southern blot analysis. The thick bars under the maps represent the probes used in this and other figures, except that the *ETat 1.2* probe used in this figure is shown as a hatched bar. In the *ETat 1.2CR* ES map, the arrows designate the two BamHI sites used for inverse PCR using the pairs of primers indicated by arrowheads, and the thick line highlights the BamHI-HpaI fragment cloned as a result of this PCR (step 1 in Figure 5). A, ApaI; Ba, BamHI; E, EcoRI; H, HindIII; Hp, HpaI; P, PstI; Sm, SmaI; V, EcoRV.

al., 1985; Kooter et al., 1987; Pays et al., 1989). As expected, in these ESs an *SRA* copy was present immediately upstream from the barren region, and this gene was not detected in the ESs transcribed in the ETat 1.2S clones (data not shown). Finally, the restriction maps of the upstream environment of *SRA* in the *ETat 1.10R* and *ETat 1.2CR* ESs appeared to be similar. Based on these results, we attempted to clone DNA fragments containing both *SRA* and *VSG*. Due to the presence of a large intervening region of repeats that prevented direct cloning, a special strategy was devised. In the *ETat 1.2CR* ES two BamHI sites, arrowed in Figure 4B, were conveniently located for this purpose. The 5′-site was mapped approximately 3 kb upstream from *SRA*, whereas the 3′-site was present in the *VSG*. The ETat 1.2CR gDNA was digested with BamHI, then ligated and subjected to inverse PCR using as pairs of primers the oligonucleotides shown by arrowheads in the *ETat 1.2CR* ES map (Figure 4B). As expected from the map, a fragment of 3.7 kb containing approximately 3.2 kb upstream from *SRA* was obtained. The nucleotide sequence of this region indicated that *SRA* was preceded by 76 bp repeats typical of *VSG* ESs (Bernards et al., 1985) (Figure 5). Upstream from these repeats, the sequence was virtually identical to that found downstream from *ESAG 5* in the *AnTat 1.3A* ES, starting precisely in the single 76 bp repeat-related element located approximately 840 bp downstream from the stop codon of *ESAG 5* (dotted underline in Figure 2 of Pays et al., 1989). These results confirmed the location of an *SRA* copy in a *VSG* ES. As expected from this location, the sequence of the *SRA* cDNA was identical to that of the gene present in the ES, and *SRA* transcription fully resisted α-amanitin (Kooter and Borst, 1984) (data not shown).

The *ETat 1.10* ES Is Severely Truncated

In order to characterize completely the *ETat 1.10R/1.2CR* ES, an inverse PCR strategy was employed (steps 2 and 3, see Experimental Procedures and scheme in Figure 5). A 4 kb fragment was amplified after EcoRV digestion of the genomic DNA from the ETat 1.2CR clone, followed by self-ligation and priming with the *ETat 1.2*-specific P121 oligonucleotide employed previously, together with different oligonucleotides specific to the region immediately downstream from the 5′-terminal BamHI site of the fragment initially cloned. Sequence analysis of this 4 kb fragment showed that it was virtually identical to the *ESAG 5* region of the *AnTat 1.3A* ES (Figure 5). The *ETat 1.2CR* ES was subsequently cloned to the promoter region by inverse PCR on HpaI-digested ETat 1.2CR genomic DNA, using proximal primers orientated toward the 5′-EcoRV and 3′-HpaI sites of the previous cloned fragment (step 3, see Experimental Procedures) This region of the *ETat 1.2CR* ES was also very similar to the *AnTat 1.3A* ES except that the RIME retroposon (see Pays et al., 1989) was missing and the two genes encoding the subunits of the transferrin receptor, *ESAG 6* and *ESAG 7*, were not in the same order (Figure 5). As expected, the nucleotide sequence in the "hypervariable" region of *ESAG 6* was found to be identical to that determined by RT-PCR on both *ETat 1.10R* and *ETat 1.2CR* RNAs, but different from that found by RT-PCR on both *ETat 1.2BS* and *ETat 1.2CS* RNAs (data not shown). These results clearly confirmed the correct identification of the cloned sequences.

The cloned *ETat 1.10/1.2CR* ES was strikingly shorter than the *AnTat 1.3A* ES and totally lacked the 20 kb region containing *ESAG 4*, *ESAG 8*, *ESAG 3*, *ESAG 2*, and *ESAG 1*. Moreover, analysis by PCR demonstrated that none of these *ESAG*s was located downstream from SRA, a result consistent with the fact that this region lacked restriction sites. In addition, both Northern blot hybridization and run-on transcription assays confirmed the absence of transcription of *ESAGs 4*, *8*, *3*, *2*, and *1* in clone *ETat 1.2CR*, whereas the transcription of these genes was clearly detected in clone *ETat 1.2CS* (data not shown). These data indicated that the *VSG* ES transcribed in R clones differs drastically from the "usual" one (either *T.b. brucei* AnTat 1.3A or *T.b. rhodesiense* ETat 1.2CS) in the number of *ESAG*s. It is probable that the deletion characterizing the *ETat 1.2CR* ES resulted from homologous recombination between 76 bp repeats flanking the *ESAG 4* to *ESAG 1* region.

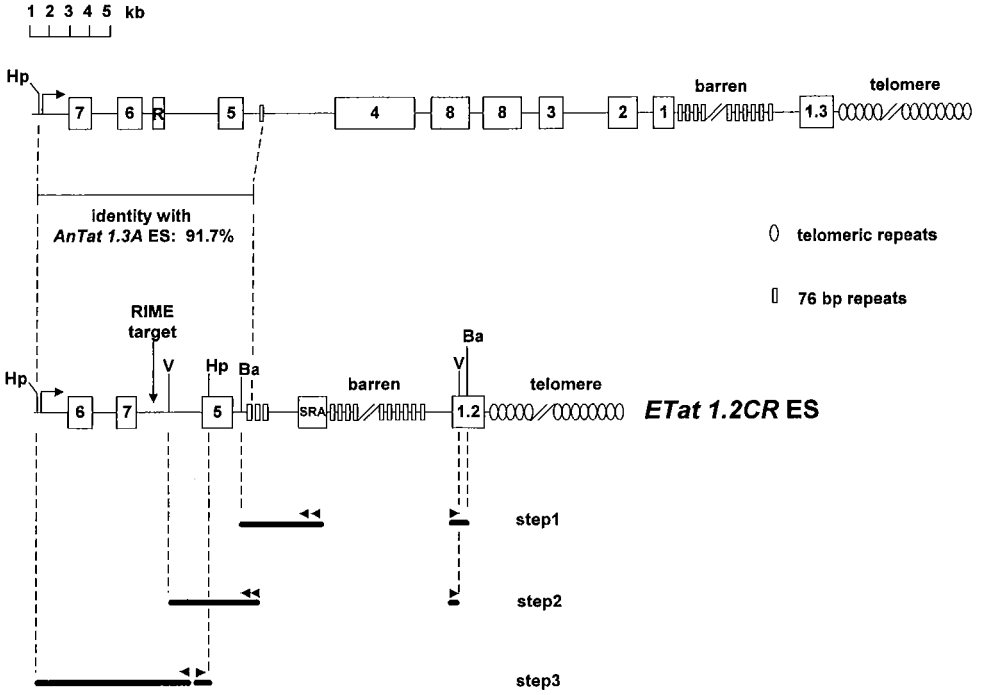

Figure 5. Comparison of the *ETat 1.2CR* and *AnTat 1.3A* ESs

The map summarizing the cloning and sequencing of the *ETat 1.2CR* ES is shown at the bottom, with indication of the primers used (arrowheads) and products obtained (thick lines) during the three successive steps of inverse PCR. This map is compared with that of the *AnTat 1.3A* ES (Pays et al., 1989), where the arrow indicates the transcription start site and the boxes represent the *VSG* and *ESAGs 1–8* as indicated by numbers, as well as the RIME retroposon (R).

Direct Evidence for the Involvement of SRA in Resistance to NHS

The possible involvement of *SRA* in resistance to NHS was evaluated by targeting of this gene into the ribosomal locus of *T.b. brucei* procyclic forms by homologous recombination. The AnTat 1 strain was chosen because it was never found resistant to NHS despite intense studies in different laboratories, and it does not contain *SRA* (data not shown). The insect-specific procyclic forms were used because they are very easy to transfect, as opposed to pleomorphic bloodstream forms. The plasmid construct used for transfection contained a gene encoding resistance to hygromycin to select the trypanosome transformants. Two independent *SRA* transformants were generated that contained either a single or a few integrated copies of the construct (data not shown). As a control, the same targeting was performed with an identical construct, but lacking *SRA*. Cyclical transmission in tsetse flies allowed the generation of bloodstream form transformants. Transcription of *SRA* was detected in these *SRA* transformants, with a mRNA longer than the wild type (2.1 kb instead of 1.5 kb) due to the presence of the 3′-UTR and polyadenylation signal of β-*tubulin* downstream from *SRA* (Figure 3). The level of this mRNA was about one-fifth that of the *SRA* mRNA in ETat 1.10R.

The resistance to NHS of the *SRA* and control transformants was measured in vitro and in mice. The presence of 5% NHS in vitro induced the lysis of the control transformants after 10 hr, exactly as for most cells of the ETat 1.2CS clone (data not shown), but the *SRA* transformants were not affected even after 22 hr (Figure 6). After 2 days of in vitro cultivation in the presence of 5% NHS, both control and *SRA* transformants were injected into mice. Even small inoculates equivalent to 1,000 parasites from the *SRA* transformants led to infection that was clearly detectable after 4 days, whereas an inoculate from the control corresponding to 100,000 parasites at the beginning of the in vitro incubation did not lead to infection even 2 weeks after injection. The transformants were also treated with undiluted NHS as described by Van Meirvenne et al. (1976). Under these conditions the same differential lysis between the control and *SRA* transformant was observed, except that complete lysis of the control occurred after only 3 hr. After 5 hr in the presence of undiluted NHS, the transformant trypanosomes were injected into mice, and results similar to those observed in the case of treatment with 5% NHS were obtained. In order to evaluate the resistance to NHS in vivo, the transformant trypanosomes were injected into mice and when the parasitaemia reached about 10^6 parasites per milliliter (around day 4), the animals were injected with 0.5 ml of NHS. The ETat 1.2CR and 1.2CS clones, as well as the *T.b. brucei* AnTat 1.1 clone, were used as controls. While the injection of NHS appeared to ablate the infection by both the ETat 1.2CS and AnTat 1.1 clones, as well as by the control transformant (no detectable parasitaemia from the day following the injection to 1 week later in the case of ETat 1.2CS, and apparently definitely in the

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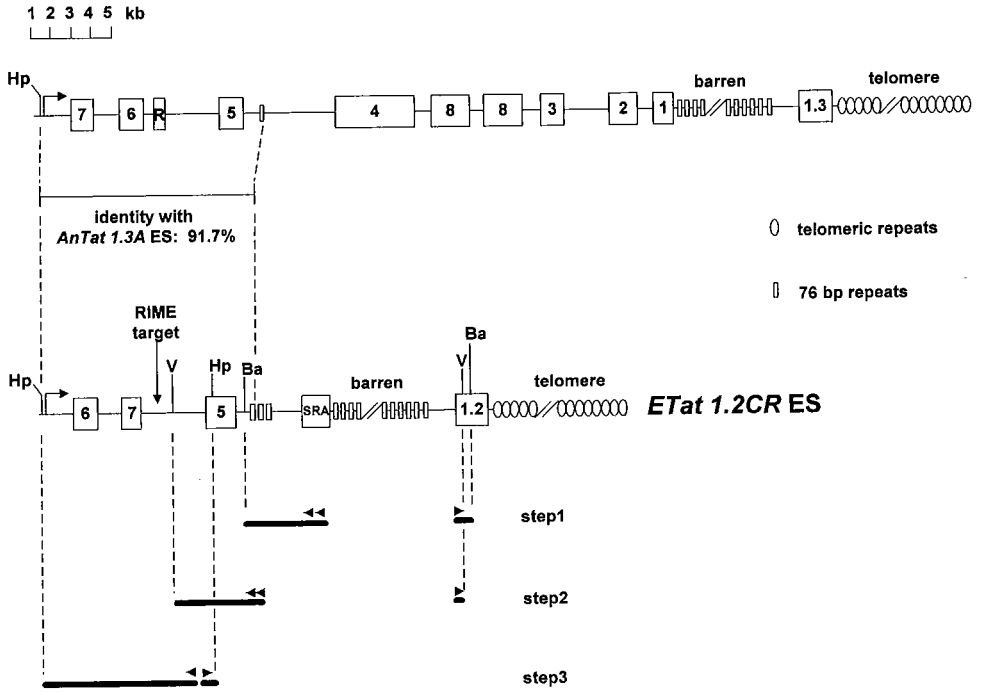

Figure 5. Comparison of the *ETat 1.2CR* and *AnTat 1.3A* ESs

The map summarizing the cloning and sequencing of the *ETat 1.2CR* ES is shown at the bottom, with indication of the primers used (arrowheads) and products obtained (thick lines) during the three successive steps of inverse PCR. This map is compared with that of the *AnTat 1.3A* ES (Pays et al., 1989), where the arrow indicates the transcription start site and the boxes represent the *VSG* and *ESAGs 1–8* as indicated by numbers, as well as the RIME retroposon (R).

Direct Evidence for the Involvement of SRA in Resistance to NHS

The possible involvement of *SRA* in resistance to NHS was evaluated by targeting of this gene into the ribosomal locus of *T.b. brucei* procyclic forms by homologous recombination. The AnTat 1 strain was chosen because it was never found resistant to NHS despite intense studies in different laboratories, and it does not contain *SRA* (data not shown). The insect-specific procyclic forms were used because they are very easy to transfect, as opposed to pleomorphic bloodstream forms. The plasmid construct used for transfection contained a gene encoding resistance to hygromycin to select the trypanosome transformants. Two independent *SRA* transformants were generated that contained either a single or a few integrated copies of the construct (data not shown). As a control, the same targeting was performed with an identical construct, but lacking *SRA*. Cyclical transmission in tsetse flies allowed the generation of bloodstream form transformants. Transcription of *SRA* was detected in these *SRA* transformants, with a mRNA longer than the wild type (2.1 kb instead of 1.5 kb) due to the presence of the 3′-UTR and polyadenylation signal of β-*tubulin* downstream from *SRA* (Figure 3). The level of this mRNA was about one-fifth that of the *SRA* mRNA in ETat 1.10R.

The resistance to NHS of the *SRA* and control transformants was measured in vitro and in mice. The presence of 5% NHS in vitro induced the lysis of the control transformants after 10 hr, exactly as for most cells of the ETat 1.2CS clone (data not shown), but the *SRA* transformants were not affected even after 22 hr (Figure 6). After 2 days of in vitro cultivation in the presence of 5% NHS, both control and *SRA* transformants were injected into mice. Even small inoculates equivalent to 1,000 parasites from the *SRA* transformants led to infection that was clearly detectable after 4 days, whereas an inoculate from the control corresponding to 100,000 parasites at the beginning of the in vitro incubation did not lead to infection even 2 weeks after injection. The transformants were also treated with undiluted NHS as described by Van Meirvenne et al. (1976). Under these conditions the same differential lysis between the control and *SRA* transformant was observed, except that complete lysis of the control occurred after only 3 hr. After 5 hr in the presence of undiluted NHS, the transformant trypanosomes were injected into mice, and results similar to those observed in the case of treatment with 5% NHS were obtained. In order to evaluate the resistance to NHS in vivo, the transformant trypanosomes were injected into mice and when the parasitaemia reached about 10^6 parasites per milliliter (around day 4), the animals were injected with 0.5 ml of NHS. The ETat 1.2CR and 1.2CS clones, as well as the *T.b. brucei* AnTat 1.1 clone, were used as controls. While the injection of NHS appeared to ablate the infection by both the ETat 1.2CS and AnTat 1.1 clones, as well as by the control transformant (no detectable parasitaemia from the day following the injection to 1 week later in the case of ETat 1.2CS, and apparently definitely in the

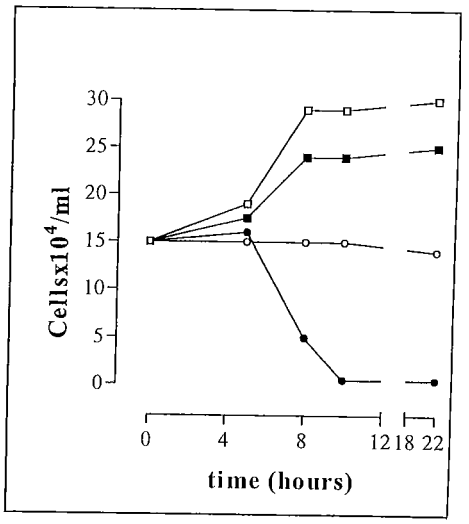

Figure 6. Tranfection of *SRA* into *T.b. brucei* Confers Resistance to NHS

T.b. brucei AnTat 1 parasites transfected with either a control plasmid (pTSARib) (dots) or PTSARibSRA (squares; two independent transformants gave identical results) were incubated at a final concentration of approximately 10^5 cells/ml in HMI-9 medium (Hirumi and Hirumi, 1989) at 37°C, in the presence (black symbols) or absence (open symbols) of 5% NHS. Under these cultivation conditions, while monomorphic parasites such as the ETat 1.2CS and 1.2CR clones grew exponentially without lag period (data not shown), pleomorphic trypanosomes such as those studied here did not proliferate within several days.

cases of AnTat 1.1 and the control transformant), the infection with the *SRA* transformants was unaffected and reached high levels (10^7 to 10^8 parasites per milliliter) in the next 2 to 3 days, similar to that observed with ETat 1.2CR. As expected from their virulence characteristics, the pleomorphic *SRA* transformants developed a 3 to 4 week parasitaemia until the mice died of infection, whereas the monomorphic ETat 1.2CR clone caused the death of the mice after 5 days. These results indicated that resistance of the *SRA* transformants to NHS not only occurred in vitro, but also in vivo. We conclude that expression of *SRA* was necessary and sufficient to confer NHS resistance to *T. brucei*.

Expression of *SRA*

SRA encodes a VSG-like protein, unusually short and apparently devoid of N-terminal signal peptide (De Greef and Hamers, 1994). Despite the generation of several different specific antibodies, both poly- and monoclonals and from mammals and chicken, the detection of this protein in R clones remained inconclusive (data not shown). In order to evaluate whether the absence of signal peptide accounts for the lack of detection as well as for the biological effect of *SRA*, we constructed a chimeric gene encoding SRA fused to the sequence for the N-terminal signal peptide of the AnTat 11.17 VSG (Do Thi et al., 1991). Transfection of this construct into *T.b. brucei* conferred resistance to NHS exactly as the wild-type *SRA*, and the chimeric SRA protein was still undetectable (data not shown).

Discussion

As predicted from serological studies (Van Meirvenne et al., 1976), our data indicate that in the ETat 1 strain of *T.b. rhodesiense* the *ETat 1.10* ES needs to be activated to obtain resistance to NHS. At least in the clone derivation that we studied, antigenic variation still allowed resistance provided the same ES was used, strongly suggesting that the genetic determinant for resistance is encoded in this site but is not the *VSG*. Evidence obtained from genomic DNA restriction mapping, cloning, run-on transcription, and RNA analysis indicated that *SRA* is an *ESAG* present only in this particular ES, in keeping with the identification of *SRA* mRNA as a transcript associated with resistance (De Greef et al., 1989). Transfection of *SRA* into *T.b. brucei* was necessary and sufficient to confer NHS resistance, demonstrating that antigenic variation-associated expression of this particular gene can confer adaptation of ETat 1 to humans.

SRA Is an *ESAG* of the *ETat 1.10* ES

Despite the presence of a very large *SRA* family, only a single member was found to be transcribed. As *SRA* encodes a VSG-like protein (De Greef and Hamers, 1989), this observation may arise because most *SRA* copies could be clustered with the majority of *VSG*s in long arrays of transcriptionally silent sequences (Van der Ploeg et al., 1982). The close association of an *SRA* copy with a *ETat 1.2* copy in a silent 3.5 kb BamHI-EcoRV fragment supports this hypothesis. In addition, *SRA* appeared to be present in a single ES, despite the existence of approximately 20 ESs in the trypanosome genome (Navarro and Cross, 1996). A similar arrangement, a *VSG* pseudogene that is embedded in the array of 76 bp repeats preceding the *VSG*, has been reported for the *221 VSG* ES (Bernards et al., 1985). This organization may not be uncommon and probably results from antigenic variation-linked recombinations occurring in this region of the *VSG* ES.

Selective Activation of the *ETat 1.10* ES in R Clones

In the ETat 1 strain the *ETat 1.10* ES was activated when trypanosomes were confronted with human serum, while a spontaneous switching to S forms was observed when NHS was removed, even if the VSG remained the same (Van Meirvenne et al., 1976; this study). Thus, in the absence of selective pressure the R phenotype seems to be counter-selected. Since this phenotype results from a defect in the uptake of the lytic factor bound to a surface receptor (Hager and Hajduk, 1997), it may be hypothesized that R clones also do not process other HDL-associated components, which could constitute a selective disadvantage. Moreover, the *ETat 1.10* ES lacks several *ESAG*s, whose function might also be important for growth. However, in vitro no difference in growth was observed between ETat 1.2CR and 1.2CS (data not shown), suggesting that neither the lack of uptake of other HDL components nor the absence of expression of *ESAG*s *1*, *2*, *3*, *4*, and *8* are required under these conditions. The nonessential character of *ESAG 1* was already noted (Carruthers et al., 1996). As is also

the case of *ESAG 4*, this may be due to the simultaneous expression of many homologous genes, termed *GRESAGs*, in other genomic loci (Pays et al., 1989; Morgan et al., 1996).

SRA Leads to Resistance to NHS

The clear phenotype observed upon transfection of *SRA* into *T.b. brucei* indicates that *SRA* is expressed. At this point we can only speculate on how this expression confers resistance. The lack of detection of the SRA protein strikingly contrasts with the very high level of the mRNA, suggesting serious problems at the level of translation and/or processing of the protein. Our results suggest that these problems are not due to the absence of N-terminal signal sequence in SRA. The presence of *SRA* transcripts, aberrant trafficking of SRA, and/or accumulation of SRA degradation products could interfere with the uptake of the lytic factor and/or its action within the cell. Since the thioprotease inhibitor leupeptin inhibits, at least partially, trypanolysis by NHS (Hager et al., 1994), it may be envisaged that SRA peptides mimic the effect of leupeptin. These hypotheses are currently under investigation.

Generality of These Observations

So far the results described here were confirmed in three other strains of *T.b. rhodesiense* (AnTAR 25, TRZ, and ITMAS221088). However, they may not apply to all cases of trypanosome resistance to NHS. First, R clones lacking *SRA* mRNA have been reported in *T.b. rhodesiense* (Rifkin et al., 1994). Second, *T.b. gambiense* lacks *SRA* (De Greef et al., 1989). The elucidation of the mechanism by which *SRA* confers resistance will probably help to understand alternative ways to achieve the same goal.

Experimental Procedures

Trypanosomes

R clones were usually coinjected in mice or rats with an equal volume of NHS. *T.b. brucei* transformants were obtained by electroporation of procyclic forms with plasmid DNA and selected for resistance to hygromycin, followed by cyclical transmission in tsetse flies, as described in Webb et al. (1997). The targeted strain (AnTat 1) was never found resistant to NHS despite intense use in several laboratories for more than 20 years.

DNA and RNA Analyses

Southern and Northern blot analysis, DNAase I sensitivity and run-on assays were conducted as in Pays et al. (1981) and Murphy et al. (1987). PFGE was performed for 17.5 hr in 1% agarose at 14°C, at 6V/cm with initial and final switch times of 0.22 s and 3.1 s and angle of separation of ±60°.

The *VSG* cDNAs were amplified by RT-PCR.

Cloning of the *ETat 1.2CR* ES was performed by inverse PCR. In step 1, the ETat 1.2RC genomic DNA was digested with BamHI, self-ligated, and used as a template for PCR with primers P121 (5′CCGGCATGCGTCTCAAGCAGTGTCAGAC3′) from *ETat 1.2* and one of the primers, R0 (5′GGGCCAGGGAGCGCCAAGG3′) or R5 (5′CCGCTCGAGGGCTCCGTTGGACGCTGCAGTT3′), from *SRA*. Unligated gDNA was used as a negative control, and the use of two alternative primers from *SRA* allowed the control for the proper length of the desired fragment. The amplification was conducted using the long expand template PCR system (Boehringer) with 1 cycle at 92°C (2 min); 10 cycles at 92°C (10 s), 63°C (30 s), and 68°C (20 min); 20 cycles at 92°C (10 s), 63°C (30 s), and 68°C (24 min with increasing time of 24 s for each cycle); and finally 1 cycle at 68°C for 15 min. In step 2, a similar procedure as for step 1 was followed, except that the ETat 1.2CR gDNA was digested with EcoRV before self-ligation, and the primers for PCR were P121 and either ESRA1 (5′CCATCACTTCTATTGCCGCCCC3′) or ESRA2 (5′GATGATACTTTTGCTGCCGCCAC3′), both designed based on the sequence obtained in step 1. PCR conditions were the same as above except that the annealing temperature was 65°C. A unique fragment of about 4.5 kb was obtained, blunt-ended, and subcloned for full sequencing. In step 3, the ETat 1.2CR gDNA was digested with HpaI before self-ligation. The primers for PCR were ESRA6B (5′CTGTTACGCCATTATTACCAGC3′) and ESRA7B (5′AATCAGGCTTTTACCGCCGTC3′), which were designed based on the sequence obtained in step 2, including a 3′-nucleotide specific to the *ETat 1.2RC* ES as determined by the comparison with known ES sequences. PCR conditions were the same as above except that the annealing temperature was 57°C. A unique fragment of about 6.5 kb was obtained, blunt-ended, and subcloned for full sequencing.

The plasmid used for targeting *SRA* into *T.b. brucei* was constructed by first replacing the procyclin promoter present in pTSA-HYG2 (635 bp Asp718-XhoI fragment) (Sommer et al., 1992) with a 2 kb HindII fragment encompassing the *T. brucei* rDNA promoter, then reintroducing the procyclin splice site (290 bp SpeI fragment) downstream from this promoter (in HindIII of pTSA-HYG2), generating pTSA-Rib. The *SRA* open reading frame, preceded or not by the sequence of the N-terminal signal peptide of the AnTat 11.17 VSG (Do Thi et al., 1991), was PCR-amplified from the cDNA (De Greef et al., 1989) with flanking XhoI and BglII sites, subcloned in a plasmid termed pEST to allow insertion, immediately downstream from the gene, of a 0.83 kb BamHI-BglII fragment containing the 3′-UTR and polyadenylation site of *T. brucei* β-*tubulin* mRNA, then the 2.2 kb *SRA*-containing XhoI-BglII fragment was inserted between the XhoI and BamHI sites located downstream from the rDNA promoter of pTSARib, generating pTSARib-SRA. The plasmids were linearized with SphI before electroporation. The correct insertion into the rDNA promoter locus was checked by Southern blot analysis (data not shown).

Acknowledgments

We thank Y. Claes (ITG, Antwerp), P. Tebabi, L. Brys, D. Nolan, S. Rolin, and L. Lecordier (Brussels) for help. This work was supported by research contracts with the Communauté Française de Belgique (ARC), by the Belgian Fonds de la Recherche Scientifique (FRSM and Crédit aux Chercheurs), by the WHO TDR program, and by the Interuniversity Poles of Attraction Programme, Belgian State, Prime Minister's Office, Federal Office for Scientific, Technical, and Cultural Affairs. L. V. is a Research Associate at the Belgian National Fund for Scientific Research.

Received August 20, 1998; revised October 28, 1998.

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