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Molecular & Biochemical Parasitology 135 (2004) 39–47
MOLECULAR & BIOCHEMICAL PARASITOLOGY

The *Trypanosoma brucei* reference strain TREU927/4 contains *T. brucei rhodesiense*-specific *SRA* sequences, but displays a distinct phenotype of relative resistance to human serum

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Received 5 January 2004; accepted 13 January 2004

Abstract

The *Trypanosoma brucei* reference strain TREU927/4 exhibits some resistance to lysis by normal human serum (NHS), but this resistance is never complete even after selection. The genome of this strain contains a minimum of eight sequences related to the *T. brucei rhodesiense*-specific serum resistance-associated gene (*SRA*), which encodes a truncated variant surface glycoprotein (VSG) conferring full resistance to lysis by NHS. We selected two sequences showing the highest similarity to *SRA* and also preceded by a region ("cotransposed region") present immediately upstream from *SRA* in the *VSG* expression site termed R-ES, where *SRA* is expressed in *T. brucei rhodesiense*. Whereas one of these sequences appears to be a pseudogene, the other, which is contained within a cluster of *VSG* basic copies (BCs), encodes a VSG truncated in the C-terminal domain. In the latter gene, an inserted region encoding surface-exposed loops similar to those of the *BoTat 1.20 VSG* interrupts the full SRA sequence. Therefore, this gene was termed *SRA-BC*, for the putative *VSG* basic copy from which *SRA* was derived. Neither this gene nor other *SRA*-like sequences appeared to be responsible for the relative resistance of TREU927/4 to NHS, since (i) transfection of *SRA-BC* in *T. brucei brucei* did not confer increased resistance; (ii) *SRA* transcripts could not be detected in this strain, even when focusing the search on the limited *SRA* sequence necessary to confer resistance and when using strain-specific *SRA* probes on RNA from cells selected in the presence of NHS.

Keywords: *Trypanosoma brucei* genome; Resistance to human serum; Sleeping sickness

1. Introduction

Trypanosoma brucei brucei and *T. brucei rhodesiense* are morphologically and physiologically indistinguishable, but the former subspecies is unable to infect humans, as opposed to the latter. Transfection of a single *T. brucei rhodesiense* gene termed *SRA* (for serum resistance-associated) was necessary and sufficient to confer human infectivity to *T. brucei brucei* [1], identifying this gene as a major, if not the sole, difference between the two subspecies. *SRA* appears to encode a truncated form of variant surface glycoprotein (VSG) [2,3], which allows trypanosomes to resist the natural lytic factor apolipoprotein L-I following interaction of its N-terminal α-helix with the C-terminal α-helix of this protein in the lysosome of the parasite [4]. *SRA* is transcribed in a particular *VSG* expression site termed R-ES, which is selected when trypanosomes are confronted to normal human serum (NHS) [1,5]. PCR analysis indicated that both *SRA* [6–8] and the R-ES [8,9] are reliable markers of *T. brucei rhodesiense*, suggesting that the mechanism by which this subspecies resists NHS generally involves SRA. Thus, as yet *SRA* is the best diagnosis tool to identify *T. brucei rhodesiense* [6–9].

The TREU927/4 strain of *T. brucei* was selected for genome sequencing. As a natural consequence of this choice, several groups in the field use this strain as preferred experimental material [10]. The detection of several *SRA* sequences in the available database [5] prompted us to investigate the following questions: (i) Is this strain human-infective, thus, dangerous to manipulate? (ii) Is *SRA* expressed in this strain? (iii) Should this strain be labelled *T. brucei rhodesiense*? We show that TREU927/4 manifests some resistance to NHS, although clearly not in the

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doi:10.1016/j.molbiopara.2004.01.004

same way as *T. brucei rhodesiense*. Moreover, TREU927/4 contains a *VSG* from which *SRA* is likely to have derived. However, despite the presence of this gene and several other *SRA*-like sequences in the genome, expression of *SRA* could not be detected.

2. Materials and methods

The TREU927/4 strain was obtained from Dr. M. Turner (Glasgow). The original stock was isolated in 1970 in Kiboko (Kenya) by Goedbloed et al. [11] from infected salivary glands of tsetse flies. These cells were passaged 3–10 times in mice before transfer to the Wellcome Centre of Molecular Parasitology (Glasgow), where the 927/4 clone was isolated [12]. DNA and RNA analyses were performed according to standard procedures. The DNA sequence around the premature stop codon of *SRA-BC* was verified by manual sequencing on both strands over a region of 500 bp.

2.1. Identification of SRA-like sequences in the genome of TREU927/4

Preliminary sequence data produced by The Institute for Genomic Research (TIGR) and the Pathogen Sequencing Unit at The Sanger Institute were analysed using the respective blast servers on the World Wide Web (http://tigrblast.tigr.org/er-blast/; http://www.sanger.ac.uk/Projects/T_brucei/). TBLASTN protocols (Wublast 2.0) [13] were carried out, querying the SRA protein sequence (SWALL:O96637) against the available nucleotide/contig databases, with no filtering of low-complexity sequences. The analysis was performed in June 2003. A total of 1050 hits were reported. Those hits were then manually screened for the presence of the domain and of the residues known to be critical to SRA function, namely the α-helix at residues 54–65 containing the key hydrophobic residues I58, L61 and I62 [4]. Eight sequences were identified, that share similarity with region spanning amino acids 32–97 of SRA, and where the residues at positions 58, 61 and 62 are conserved. These sequences are the following (respectively name of the contig, relevant region in base pairs, % amino acid identity, % amino acid similarity, *E*-value and relevant region in amino acids): TRYP10.0.000978, 15,015–14,662, 23%, 38%, 2.7E−69; 38–158; TRYP10.0.000765, 21,084–20,668, 28%, 47%, 1.9E−09; 3–144; TRYP9.0.002326*, 1542–2024, 71%, 79%, 1.2E−103, 1–161; TRYP9.0.000727*, 137,324–137, 806, 71%, 79%, 7.9E−102, 1–161; TRYP9.0.000462, 23,707–23,183, 26%, 40%, 4.9E−09, 31–213; chr6/30P15/44, 12,742–13,165, 22%, 38%, 2.4E−05, 28–176; chr8/27P2/100, 63,824–63,387, 31%, 51%, 2.2E−31; 10–155; chr8/27P2, 111,596–111,141, 63%, 78%, 2.2E−113, 3–154. (It is likely that the sequences marked with ∗ correspond to a single locus.)

2.2. Plasmid constructions

The *SRA-BC* pTSARib construct was generated as follows. A pair of primers (forward 5′CTCGAGATGCCCCGAAATTCGGGCCGGACAAC and reverse 5′GATATCTAGATAAGTGTTAAAATATATCATAGTATATCAATTTGTAG) were used to amplify the full *SRA-BC* open reading frame, together with the sequence of the predicted 3′-untranslated region of its mRNA, by PCR on TREU927/4 DNA. The resulting 1670 bp-fragment was digested by *Xho*I and *Xba*I (underlined in the primer sequences) and cloned by ligation in the *Xho*I + *Xba*I digested vector pTSARib, which contains a 1.9 kbp *Hin*dII fragment from the 5′-region of the *T. brucei brucei* ribosomal genes, as well as a gene encoding resistance to hygromycin [1]. This ligation resulted in the insertion of *SRA-BC* between the trans-splicing signal of the procyclin genes and the polyadenylation signal of *α-tubulin*. The plasmid construct was targeted by electroporation into the ribosomal locus of *T. brucei brucei* AnTaR1 procyclic forms, following linearization by *Sph*I digestion. The transformants were selected by incubation in the presence of 25 μg/ml hygromycin, and then cyclically transmitted through tsetse flies to obtain bloodstream forms.

2.3. Primers

The primers for PCR and RT-PCR focused on the 300-bp essential region of *SRA* were: forward 5′CCGCCTTTGACGAAGAGCCCGTC (nucleotides 89–111 from the initiation codon) and reverse 5′CTGAAATGTGCCCACTGGTTTTGGC (nucleotides 394–370).

3. Results

3.1. The TREU927/4 strain displays some resistance to NHS

TREU927/4 trypanosomes were injected into NMRI mice together with foetal calf (FCS) or human serum (NHS), in which they were pre-incubated for various periods at room temperature. The results are summarized in Table 1, and representative growth curves are illustrated in Fig. 1. As shown in Fig. 1A, with FCS parasitaemia was detectable after 3 days, but with NHS it appeared only after 9 days, and in only 34% of the injected mice (average from 47 infections in six independent experiments). In the latter case, if NHS was reinjected at day 12, the parasites disappeared to emerge again approximately 10 days later. Alternatively, if the blood from mice at day 12 was injected in naïve animals, again after incubation in either FCS or NHS, the infection was detectable after day 14 (Fig. 1B). Successive passages with NHS did not increase the percentage of resistant trypanosomes (34, 32, 11 and 12% positive mice at first, second, third and fourth passage, respectively: see Table 1). This infection pattern clearly contrasted with that observed

Table 1

Injected material[a]	Total mice number	Positive mice	Negative mice[b]	Positive mice (%)
TREU927/4 + FCS	11	11	0	100
TREU927/4 + NHS[c]	47	16	31	34
TREU927/4 + NHS 2d passage[d]	25	8	17	32
TREU927/4 + NHS 3d passage[d]	9	1	8	11
TREU927/4 + NHS 4th passage[d]	8	1	7	12
ETat 1.2R + NHS[e]	10	10	0	100
AnTat 1.3A Rib-SRA + NHS[e,f]	10	10	0	100
AnTat 1.3A + NHS[e,f]	10	0	10	0
EATRO1125 Rib-SRA + NHS[e,f]	10	10	0	100
EATRO1125 Rib-0 + NHS[e,f]	10	0	10	0
EATRO1125 Rib-SRA-BC + NHS[e,f]	10	0	10	0

[a] 5×10^6 trypanosomes in 50–100 μl of blood were incubated for 30 min with and then coinjected with 300 μl of serum.

[b] Negative mice: mice where parasitaemia was followed for 50 days without detection of trypanosomes.

[c] Total of six independent experiments varying preincubation times (5, 15, 30, 60 or 120 min) and volumes of serum (250 or 400 μl with 30 min preincubation). In all these experiments a similar ratio of positive mice (25–40%) was observed.

[d] Blood was harvested from a positive mouse from the previous passage, incubated with serum as described and reinjected in several mice.

[e] All these trypanosomes infect 100% of the mice when coinjected with FCS in similar conditions. Several other antigenic variants from the EATRO 1125 and 427 strains were co-injected with NHS, and parasitaemia was followed for up to 2 months, without any evidence of infection (data not shown).

[f] AnTat 1.3A bloodstream forms or EATRO1125 procyclic forms were transfected with either the pTSARib-SRA plasmid expressing *SRA*, or the empty pTSARib-0 plasmid as a control. After selection the procyclic forms were subjected to cyclical transmission in tsetse flies to obtain the corresponding bloodstream forms.

with *T. brucei brucei*, irrespective of the strain. In that case, in the presence of NHS parasitaemia could never be detected even after several months (representative result shown for the strain AnTAR1 in Fig. 1C).

When the same number of *T. brucei rhodesiense* ETaR 1 parasites was injected together with NHS in the same strain of mice, infection was detectable after 2 or 8 days, depending on the clones (Fig. 1D and E). In that case the differential timing of parasitaemia could be ascribed to the transcription status of the R-ES, which contains *SRA*. Indeed, depending on whether this ES is already active or has to be activated in the injected clone, parasitaemia appeared to be early or late [1,4] as illustrated with the infection patterns of the SRA^+ ETat 1.2R and the SRA^- ETat 1.2S clones in Fig. 1D and Fig. 1E, respectively. This observation contrasts with the systematic lag (9–13 days) observed with TREU927/4 cells injected with NHS, irrespective of whether the parasites were already pre-selected in NHS. Thus, resistance to NHS in TREU927/4 clearly differs from that in *T. brucei rhodesiense*: in the former case it would seem as if always only a minority of cells survive after incubation in NHS.

The growth phenotype of TREU927/4 was also analysed in vitro. As shown in Fig. 2, these cells were lyzed by NHS, but the kinetics of this process was slower than that of either the *T. brucei rhodesiense* NHS-sensitive clone ETat 1.2S (Fig. 2) or *T. brucei brucei* clones (not shown). Clearly, this phenotype contrasted with the high proliferation rate of NHS-resistant *T. brucei rhodesiense* ETat 1.2R.

In conclusion, TREU927/4 trypanosomes showed increased resistance to NHS as compared to either *T. brucei brucei* or *T. b rhodesiense* sensitive clones, but this resistance was always incomplete, even if selected for.

3.2. Presence of SRA-like sequences in the TREU927/4 genome

Based on the sequence data currently available from The Institute for Genomic Research and The Wellcome Trust Sanger Institute, we estimated that a minimum of eight SRA-like sequences are present in the genome of TREU927/4 (see Section 2). In particular, we selected two sequences clearly distinguishable from the others given their highly significant *E*-values. These sequences not only shared the highest level of identity (around 70%) with the region of *SRA* that encodes the essential N-terminal α-helix [4], but also showed considerable sequence conservation with the *T. brucei rhodesiense* R-ES sequence located immediately upstream from the gene, a region often termed “cotransposed region”. In one case (chr8/27P2, nt 111,596–111,141; accession numbers of the two overlapping clones: AQ 945788 and AQ 638893) the comparison was limited due to incomplete coverage by the available sequences. Nevertheless, it was clear that the region contained a pseudogene since the *SRA* initiation codon was mutated into ATA, and no other alternative open reading frame could be detected (data not shown). The second case (TRYP9.002326, nt 1542–2024) was more interesting since a clear interpretation could be drawn from the sequence comparison. As summarized in Fig. 3, the region 137,096–140,470 of the 157,340 bp sequence reported under the accession number AC 007926 appeared to contain a *SRA* homologue included within an array of *VSG* basic copies (BCs). This gene and its immediate environment exhibited all the typical characteristics of *VSG*s, including the 18 nt-signature present in the 3′-untranslated region of *VSG* mRNAs as well as the *VSG*-like sequence of *SRA* [2,3]. In-

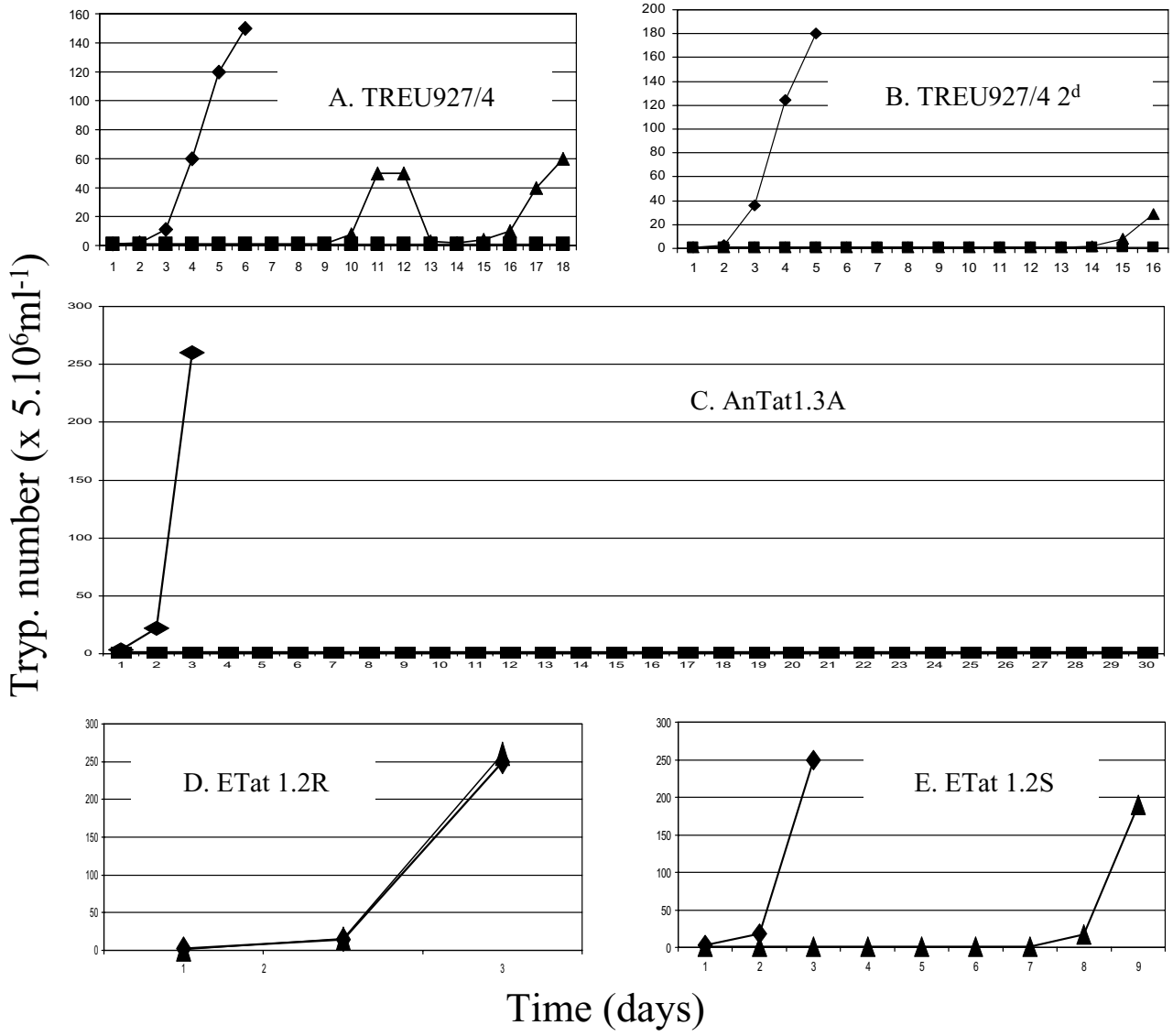

Fig. 1. Resistance to NHS in different *T. brucei* strains. NMRI mice were injected with parasites in the presence of either FCS or NHS. The parasitaemia curves are shown for one mouse representative of each of the categories listed in Table 1. The details of infection are given in the footnotes of Table 1. Triangles = positive mice with NHS; squares = negative mice with NHS; diamonds = positive mice with FCS.

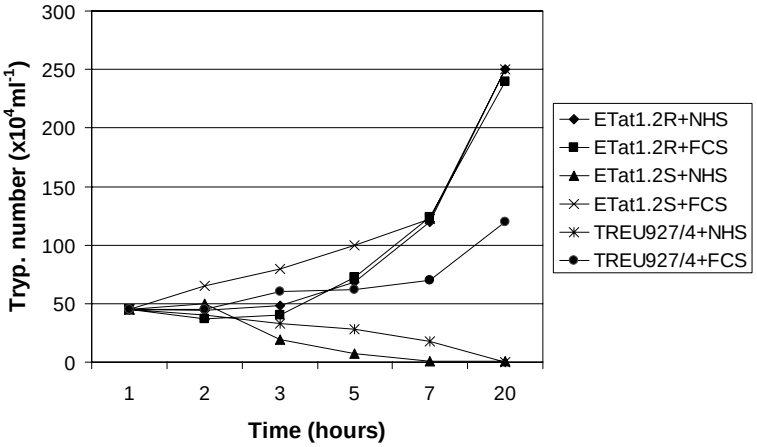

Fig. 2. Growth curves of different *T. brucei* strains incubated in vitro in the presence of either FCS or NHS (same conditions as in [1,4]).

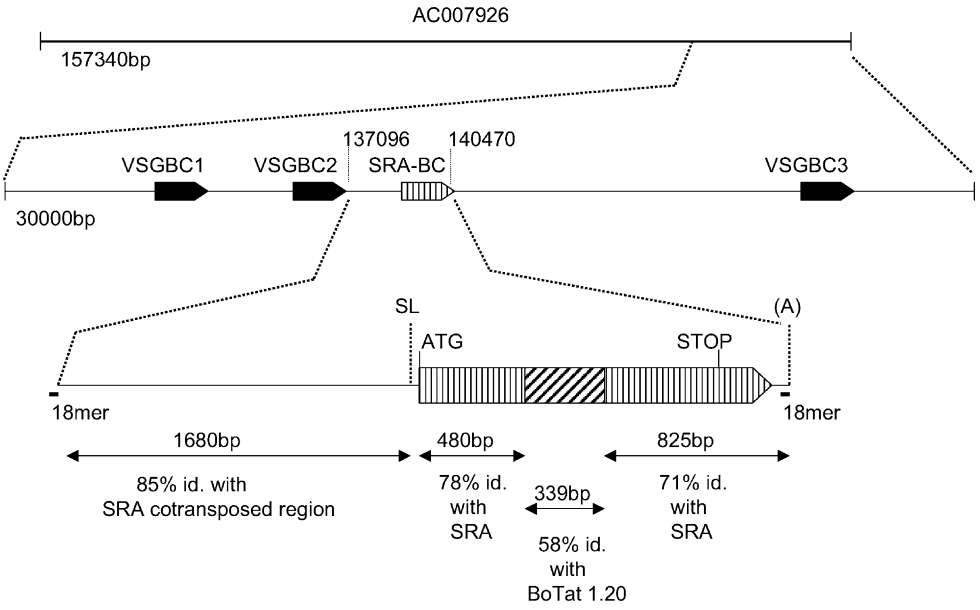

Fig. 3. *SRA-BC* in TREU927/4. A *SRA*-like gene including an insert encoding VSG surface-exposed loops is contained within an array of *VSG* basic copies (arrowed boxes). 18mer = conserved stretch of the 3′-UTR of *VSG* mRNAs; SL = spliced leader acceptor site; ATG = initiation codon; STOP = stop codon; (A) = polyadenylationsite.

terestingly, this gene was longer than *SRA* and showed the normal size of *VSG* sequences, due to the insertion of an additional 339 nt-stretch approximately 480 nt downstream from the initiation codon. However, the open reading frame only contained 452 amino acids due to the truncation of the C-terminal domain. Thus, the protein coded by this gene should lack the C-terminal glycosylphosphatidylinositol anchor sequence (Fig. 4). Since the DNA sequence specific for the typical C-terminal end of VSGs as well as that of *VSG* mRNA 3′-UTR was present in *SRA-BC*, we checked if

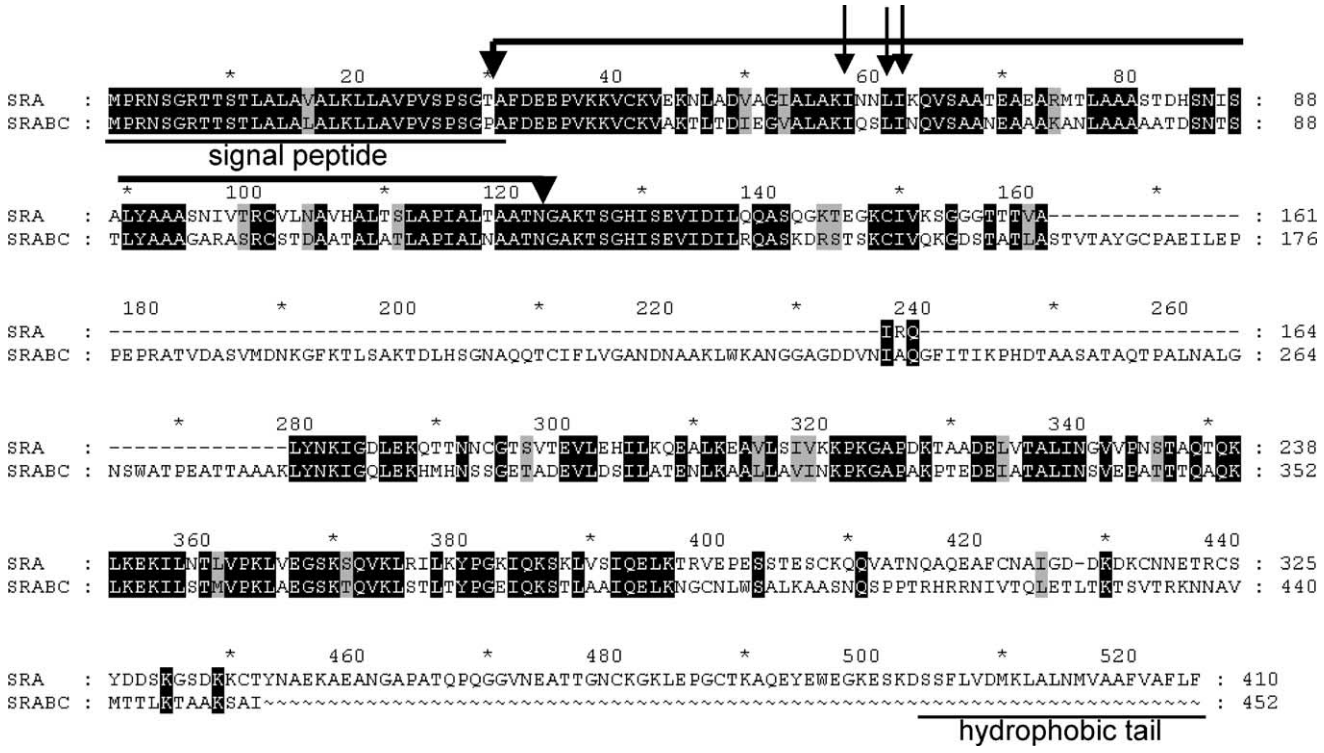

Fig. 4. Amino acid sequence alignment between SRA and SRA-BC. Dark and grey boxes, respectively, identify identical and similar residues. The putative N-terminal signal peptide and C-terminal hydrophobic tail are underlined. The arrowed upper bar identifies the gene regions amplified by PCR to generate the hybridization probes for the experiments described in Fig. 8, and the arrows designate key amino acids involved in the function of SRA [4].

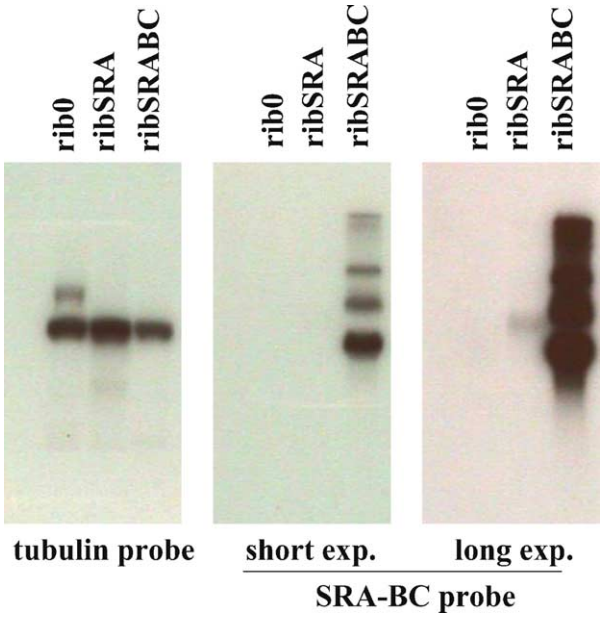

Fig. 5. *SRA-BC* expression in EATRO1125 *T. brucei brucei* cells transfected with various pTSARib constructs in the rDNA locus [1]. Northern blots of total RNA (10 μg) from transfected trypanosomes (first lane: control pTSARib-0; second lane, pTSARib-SRA; third lane: pTSARib-SRA-BC) were hybridised with either *α-tubulin* or a *SRA-BC* probe, as indicated. In the last panel, a longer exposure is shown to reveal the cross-detection of *SRA* transcripts in the second lane. The ladder pattern of transgene transcripts is typical of this construct in this genomic target [1,4].

the premature stop codon found in the genome database was genuine, and indeed confirmed this observation by manual sequencing on both strands. The 339 bp insert showed approximately 58% sequence identity with the corresponding region of the *BoTat 1.20 VSG*. Based on all its characteristics, we termed this sequence *SRA-BC*, for the *VSG* basic copy from which *SRA* was probably derived. Interestingly, the alignment between SRA-BC and SRA (Fig. 4) indicated that the region missing in the latter was between amino acids 160 and 270, a region that includes the surface-exposed domain of VSGs and of the VSG-derived transferrin receptor, roughly between amino acids 170 and 270 [14–17].

3.3. SRA-BC does not confer resistance to NHS

SRA-BC and its environment, from the mRNA initiation codon to the polyadenylation region, was amplified by PCR and cloned in the pTSARib vector, that allows insertion and ectopic expression of genes in the ribosomal locus of *T. brucei brucei* [1,4]. As described previously [1], the *SRA-BC* pTSARib construct was transfected into *T. brucei brucei* procyclic forms, which were cyclically transmitted in tsetse flies to obtain transformant bloodstream forms. As shown in Fig. 5, transcription of *SRA-BC* occurred in these transformants. The pattern of *SRA-BC* transcripts was very similar, both qualitatively and quantitatively, to that of *SRA* or *SRA* derivatives in our previous work [1,4]. Nevertheless, in clear

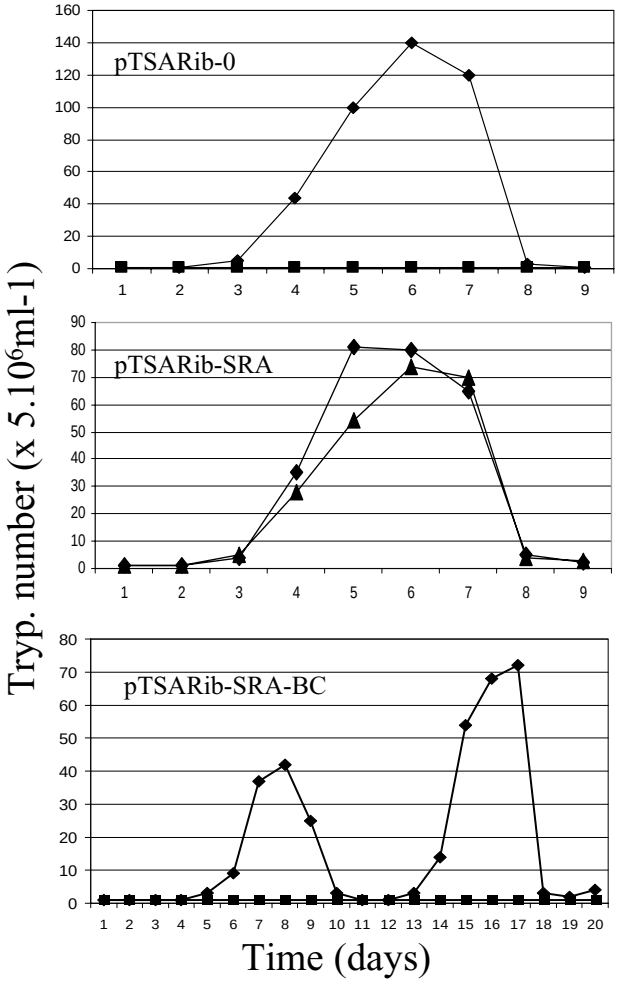

Fig. 6. Parasitaemia patterns of three different transgenic *T. brucei brucei* lines. Pleomorphic EATRO1125 *T. brucei brucei* cells with insertion of various pTSARib constructs in the rDNA locus [1] were injected into NMRI mice in the presence of either FCS or NHS, as indicated. See Table 1 for details of infection. Triangles = positive mice with NHS; squares = negative mice with NHS; diamonds = positive mice with FCS.

contrast with the results reproducibly obtained with *SRA*, the *SRA-BC* transformants were unable to resist NHS (Fig. 6).

3.4. SRA is not expressed in NHS-resistant TREU927/4 cells

DNA probes were generated by PCR to cover the entire *SRA*-related sequence of the two selected homologues from the TREU927/4 strain. These probes were able to recognize several fragments in high stringency-Southern blot analysis of the TREU927/4 DNA, and the fragments expected from the restriction maps of the known sequences were all detected (data not shown). These probes were used to search for *SRA*-like transcripts in TREU927/4 trypanosomes grown with either FCS or NHS. The Northern blot data in Fig. 7 did not reveal such transcripts, despite the fact that both probes clearly cross-hybridized with the *SRA* mRNA from the *T. brucei rhodesiense* ETat 1.2R clone.

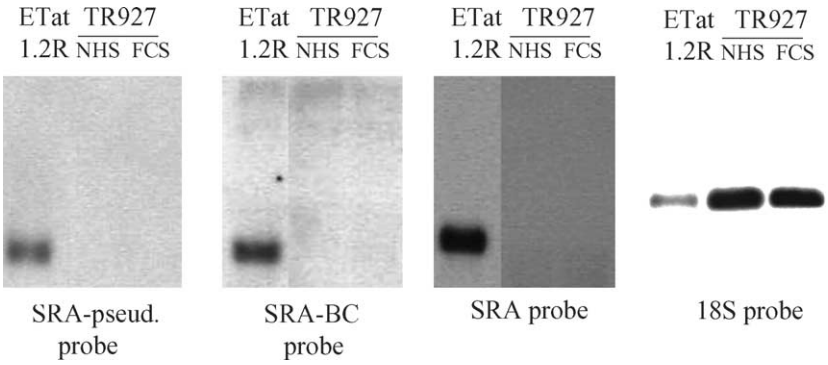

Fig. 7. Absence of *SRA* transcripts in TREU927/4. Northern blots of total RNA (10 μg) from either *T. brucei rhodesiense* ETat 1.2R or TREU927/4, grown in mice with either FCS or NHS, were hybridised with various probes, as indicated.

In order to check if the expression of only a limited sequence of an *SRA*-like gene was responsible for the relative resistance to NHS in TREU927/4, we used PCR to amplify a 300 bp fragment centred to the region of *SRA* known to be essential for the phenotype conferred by this gene [4]. Two stretches totally conserved between *SRA* and *SRA-BC*, respectively located at nt 89–111 and 370–394 downstream from the *SRA* initiation codon, were used as primers. In a first set of experiments, the amplified fragments from the two genes were used as probes for Southern blot analysis of TREU927/4 DNA. As shown in Fig. 8, under high stringency no significant hybridisation was obtained with the *SRA* probe, whereas the expected fragments of *SRA-BC* were detected with their specific probe. In a second set of experiments, the primers were used to amplify TREU927/4 sequences, by either PCR or RT-PCR. In the former case the products were cloned and analysed individually by either sequencing or digestion with specific restriction endonucleases. All 20 analysed products were derived from the *SRA-BC*. Moreover, RT-PCR conducted with these primers on TREU927/4 RNA was consistently negative, whether performed on RNA from cells grown with or without NHS.

We conclude that none of the *SRA*-like sequence present in TREU927/4 is transcribed, even in the presence of NHS.

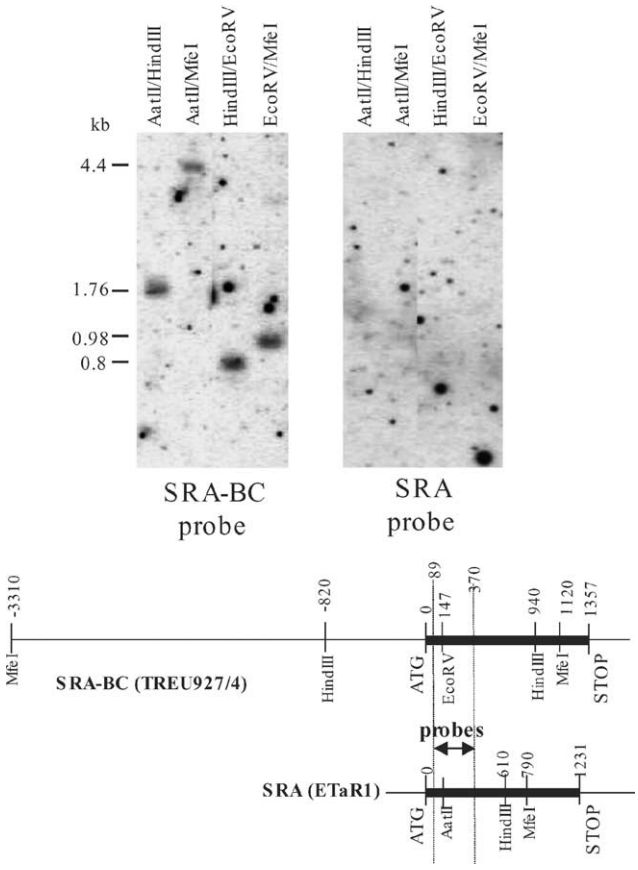

Fig. 8. Absence of core *SRA* sequence in TREU927/4. The scheme shows the restriction maps of *SRA-BC* and *SRA*, with indication of the region amplified by PCR in both cases (between dotted lines) to generate specific hybridisation probes (double arrowed region). The hybridisation pattern obtained with the two probes is shown for the *Eco*RV + *Hin*dIII, *Eco*RV + *Mfe*I, *Aat*II + *Hin*dIII and *Aat*II + *Mfe*I digests. *Eco*RV and *Aat*II are specifically found at the same locus in the *SRA-BC* and *SRA* sequence, respectively. The *Hin*dIII and *Mfe*I sites are conserved in both *SRA-BC* and *SRA*.

4. Discussion

As is typically the case for *T. brucei rhodesiense*, but not for *T. brucei brucei* or *T. brucei gambiense* strains [1,6–9], the genome of TREU927/4 contains several members of the *SRA* family. This includes at least two members showing extensive homology with the *T. brucei rhodesiense SRA* arrangement not only in the *SRA* open reading frame, but also in its 5′-environment. Based on these considerations, it is obvious that the reference strain of *T. brucei* is related to *T. brucei rhodesiense*. TREU927/4 also shows some resistance to NHS, but the pattern of this resistance is quite different from that of *T. brucei rhodesiense*, at least in the ETaR strain. It exhibits a systematic lag-period that does not exist in *T. brucei rhodesiense*, suggesting a different mechanism for resistance. In particular, this lag cannot be ascribed to the need for activation of a particular *VSG* ES as is the case in *T. brucei rhodesiense* [1], since injection into mice of cells preselected in NHS did not lead to a

faster appearance of parasites in the blood. It seems as if only a minority of TREU927/4 cells are able to survive in NHS, but these cells would be unable to grow in its presence so that it is only after NHS clearance from the blood that parasitaemia could develop. Second, TREU927/4 cells do not survive in the continuous presence of NHS in vitro, although their lysis is significantly delayed if compared to *T. brucei*. Third, we were unable to select for full resistance to NHS, suggesting that this phenotype is constitutively incomplete. Finally, none of the *SRA*-like sequences of TREU927/4 seems to be expressed, even under NHS selective pressure, contrasting with the clear *SRA* induction by NHS in *T. brucei rhodesiense* [1]. Therefore, TREU927/4 contains *T. brucei rhodesiense*-specific sequences, but does not exhibit the *T. brucei rhodesiense*-specific mechanism of resistance to NHS.

Whatever the origin of the TREU927/4 genome, its study led to the discovery of a probable ancestor of *SRA*. This gene, termed *SRA-BC*, is a *VSG* as determined by the analysis of the genomic context (array of *VSG* BCs) and DNA sequence that contains all characteristics of *VSG*s. However, the open reading frame of this gene is interrupted by a premature stop codon, so that the C-terminal end is lacking. Interestingly, in this gene the *SRA* sequence contains the insertion of a *BoTat 1.20 VSG*-like sequence predicted to code for the surface-exposed domain of the antigen. Therefore, it can be hypothesized that SRA evolved from SRA-BC by the deletion of the surface-exposed domain. This conclusion is in full agreement with those derived from structure modelling [3]. Thus, the deletion of this domain may have been required to allow SRA to confer resistance to NHS. Since the intracellular routing of SRA strikingly differs from that of VSG, being mainly targeted to the lysosome instead of the plasma membrane [4], it is likely that the truncation was necessary for proper intracellular localization of the resistance factor.

While we demonstrated experimentally that *SRA-BC* could not confer resistance to NHS, this potential cannot be excluded for other members of the *SRA* family present in the TREU927/4 genome. However, a careful search for *SRA* transcripts using TREU927/4-specific *SRA* probes in both RT-PCR and Northern blot analysis, did not reveal *SRA* expression, even from only partial regions and even in cells selected in the presence of NHS. Therefore, we conclude that a mechanism different from that of *SRA* is at work in TREU927/4 to allow these cells to develop partial resistance to NHS. A similar conclusion holds for *T. brucei gambiense*, which fully resists NHS without *SRA* [1,8].

The reasons for the lack of *SRA* expression in TREU927/4 can only be speculated at this stage. The *SRA-BC*, like other *VSG* BCs, is presumably located in an environment totally incompetent for transcription [18]. It is tempting to propose that the other *SRA* sequence that we selected, the *SRA* pseudogene (ATG initiation codon mutated into ATA) immediately flanked by a R-ES-like sequence, is indeed a non-functional homologue of *T. brucei rhodesiense SRA*. It is possible that this sequence is contained in a *VSG* ES, which would be consistent with its incomplete representation in the genome database. Indeed, the *VSG* ESs are refractory to cloning, and are significantly under-represented in the current database [5]. An alternative explanation for the lack of *SRA* expression in TREU927/4 would be the absence, or major rearrangement, of the R-ES, since the diagnostic PCR for this site was negative in this strain [8].

This work clearly demonstrated that TREU927/4 should be regarded as possible human pathogen. Therefore, we urge the research community working on trypanosomes to handle this *T. brucei brucei* reference strain with particular caution.

Acknowledgements

We thank Dr. D. Nolan for comments on the manuscript. Sequencing of the *T. brucei* genome is accomplished as part of the *Trypanosoma* Genome Network with support by The Wellcome Trust and by an award from the National Institute of Allergy and Infectious Diseases, National Institute of Health. This work was supported by the “Fonds de la Recherche Scientifique Médicale” 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.

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