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The expression of the major shed *Trypanosoma cruzi* antigen results from the developmentally-regulated transcription of a small gene family

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To better understand the mechanisms involved in the developmental expression of *Trypanosoma cruzi* antigens we examined the gene structure and transcription properties of the major shed trypomastigote antigen (SAPA). We report in this paper that SAPA is encoded by a small family of at least 6 genes which differ mainly in the length of a repeat region made up of tandemly arranged 36-bp repeat units. SAPA genes are located distant from chromosomal telomeres as inferred from their insensitivity to Bal31 nuclease treatment. Furthermore, Northern blot and S1 protection analyses strongly support the fact that most (or all) SAPA genes are transcribed in the infective form of the parasite.

Shed antigen; Gene family; Telomere; *Trypanosoma cruzi*

1. INTRODUCTION

Trypanosoma cruzi, the agent of the American trypanosomiasis Chagas' disease, multiplies in mammals as intracellular amastigotes which differentiate in trypomastigotes that are released into the mammalian bloodstream after the disruption of the parasitized cells. The antigenic molecules located on the trypomastigote surface have been widely studied, since this form of the parasite is responsible for the invasion of distant tissues and is exposed to the attack of the immune system. Several genes encoding antigenic proteins which bear repetitive epitopes have been cloned and characterized, some of which were found to be specific to the trypomastigote stage of the parasite [1–3]. We have reported the isolation from a *T. cruzi* expression library of a clone encoding antigenic determinants present on a protein that is shed by trypomastigotes during the early period of infection [4]. Remarkably, this is the main antigen recognized by sera from acute [4] and congenital [5] cases of Chagas' disease, and due to its properties we named it SAPA (shed acute phase antigen) [4]. Sequence analysis showed that the coding region of SAPA clone is made up of tandemly arranged 36-bp repeats flanked by regions of non-repetitive DNA [4].

Little is known about the mechanisms responsible for the expression of *T. cruzi* antigens. It has recently been described as a multigene family, encoding a trypomastigote 85-kDa antigen, which shows some features in common with variant surface glycoprotein (VSG) gene families of African trypanosomes [6]. The finding that the only member of this family that seemed to be transcriptionally active was telomeric, led to the suggestion that *T. cruzi* might have retained or acquired the mechanisms of antigenic variation [6]. In this paper we show that, unlike the 85-kDa antigen, SAPA is encoded by a small gene family whose members differ in the number of repeat units and most (or all) are transcribed in the infective form of the parasite although they are located distant from chromosomal telomeres.

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2. MATERIALS AND METHODS

The *T. cruzi* strain used in this study was clone CA1-65 [7]. Trypomastigotes were obtained from infected Vero cell cultures, whereas epimastigotes were grown in liquid medium as previously described [1]. Nuclear DNA and total RNA purification as well as Southern and Northern blot hybridizations were performed as described [1]. SAPA clone was isolated from a λ gt11 library and sequenced [4]. A genomic library constructed in the *Eco*RI site of λ gtWES-λ B DNA was screened using SAPA clone as probe and 3 recombinant phages were isolated. Clones 41, 42 and 28 are restriction fragments derived from independent phages which were subcloned in pUC19 and characterized by restriction mapping and cross-hybridization. Clones SAPA and 28 correspond to overlapping genomic regions. To determine the length of the repeat region present in clones 41, 42 and 28, the DNA inserts were end-labelled and partially digested with *Rsa*I which cuts once each 36-bp repeat [4]. The boundaries of the repeat regions were confirmed by nucleotide sequencing starting from the conserved *Pvu*II and *Hae*II restriction sites. Digestion of nuclear DNA with Bal31 nuclease was carried out incubating 30 μg of DNA in the presence of 2 U of Bal31 at 30°C in 30 μl of reaction volume. Aliquots were taken after 1, 5, 20 and 30 min and the reaction stopped with EGTA (20 mM final concentration). The DNA samples were digested with *Pst*I and analyzed by Southern blot hybridization. The extent of Bal31 digestion was estimated from the decrease in length of *Hin*dIII-generated fragments of λ DNA included

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in the *T. cruzi* DNA sample. For S1 protection experiments, 20 ng of unlabelled double-stranded DNA probe was mixed with 10 μg of total trypomastigote RNA in 25 μl of hybridization buffer described by Berk and Sharp [8]. Three reactions were set up: one containing only DNA, one containing DNA and S1 nuclease and one containing DNA, RNA and S1. The mixture was heated at 85°C for 10 min and then incubated for 3 h at 63°C. The temperature of the hybridization step was estimated according to the G·C content of the probe [9]. The samples were diluted with 250 μl of ice-cold S1 reaction buffer [8] and digested at room temperature for 1 h with S1 nuclease (200 U/ml). The products of S1 protection experiments were analyzed on 2% (w/v) alkaline agarose gels, followed by Southern blotting and hybridization [10]. The double-stranded DNA probe was a 2-kb *Pvu*II-*Hae*III restriction fragment corresponding to the repeat region of clone 41 (see Fig. 2A).

3. RESULTS AND DISCUSSION

3.1. *Genomic organization of SAPA genes*

In order to identify genomic DNA fragments homologous to the SAPA clone, a Southern blot analysis was performed. The SAPA probe recognized 4-6 bands in different restriction enzyme digestions of trypomastigote nuclear DNA (Fig. 1A). When the DNA was digested with *Pst*I, 6 bands of 7, 5.5, 4.8, 4.3, 3.7 and 2.8 kb were detected (Fig. 1, lane d). These bands progressively decreased in length by further treatment with *Hpa*II and *Hae*III (Fig. 1, lanes e and f). The 4 bands obtained by *Pst*I-*Hae*III digestion (lane f) could also be detected performing double digestions with *Pst*I-*Hha*I and *Pst*I-*Alu*I (not shown). Due to the absence of restriction sites for these enzymes in the SAPA repeat sequence [4], it is likely that they define the boundaries of at least 4 discrete repeat regions. To establish whether the 6 hybridization fragments generated by *Pst*I digestion represented multiple copies of the SAPA gene, blots of *Pst*I-treated DNA were reacted with two probes corresponding to DNA sequences flanking the repeat region of SAPA clone. One of these probes was a 750-bp *Pst*I-*Pvu*II fragment containing the 5′ end of the SAPA clone and the other, a

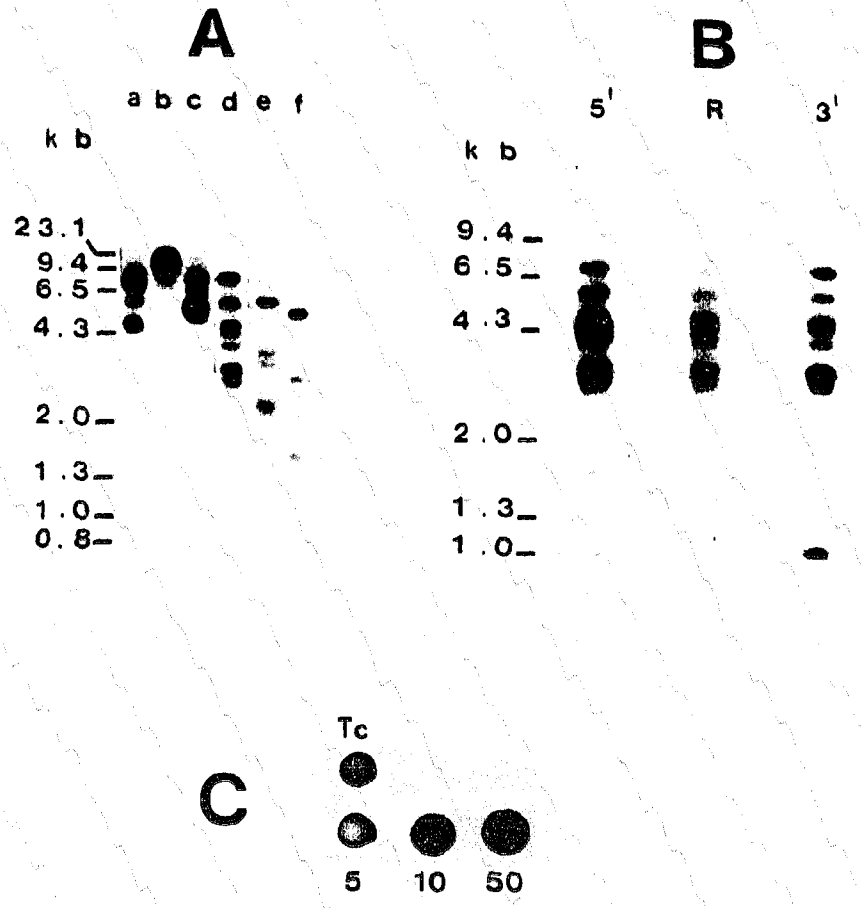

Fig. 1. Genomic organization of the SAPA gene. (A) Southern blot analysis of trypomastigotes DNA. Nuclear DNA was digested with *Bam*HI (a), *Hin*dIII (b), *Eco*RI (c), *Pst*I (d), *Pst*I-*Hpa*II (e) and *Pst*I-*Hae*III (f), blotted onto nitrocellulose filters and hybridized to the SAPA clone. The molecular weight marker (*Hin*dIII-digested λ DNA and *Hae*III-restricted Φ X 174 DNA) is represented on the left of the figure. (B) Genomic DNA was digested with *Pst*I, blotted and hybridized to restriction fragments corresponding to the repeat region (R), the 5′ end (5′) and the 3′ end (3′) of the SAPA clone. Molecular weight markers as in (A). (C) Copy number of SAPA genes in the *T. cruzi* genome, 5 μg of trypomastigote DNA (Tc) was dot-blotted together with different amounts of a plasmid containing the 3′ end of the SAPA clone (*Hae*II-*Ssp*I fragment) which represent 5, 10 and 50 copies of this sequence per genome. The filter was hybridized to the 3′ end probe.

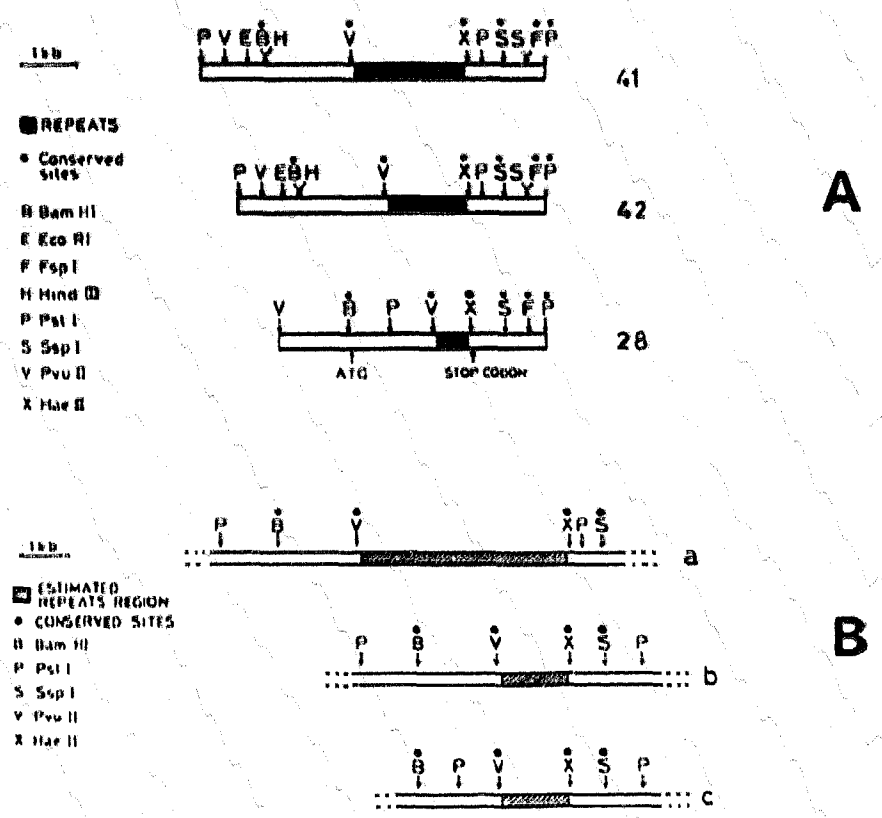

Fig. 2. Comparison of the gene structure of the members of SAPA gene family. (A) Restriction maps of genomic clones 41, 42 and 28. (B) Restriction maps of the genomic regions spanning the *Pst*I restriction fragments of 7 kb (a), 5.5 kb (b) and 3.7 kb (c) that hybridized with the SAPA clone.

600-bp *Hae*II-*Ssp*I fragment containing the 3′ end [4]. Both probes detected the same DNA bands as those recognized by the repeat probe (Fig. 1B). The hybridization with the 3′ end probe showed in addition a band of 1 kb that corresponds to a *Pst*I fragment located downstream of the 4.8 kb and 4.3 kb bands (see Fig. 2 for a restriction map of clones 41 and 42 which contain these *Pst*I fragments). The 5′ end probe also detected an extra but faint band of 1.7 kb that may represent a DNA fragment not related to SAPA genes, which shows a certain degree of homology with the probe (Fig. 1B). The comparison of the hybridization intensity of 5 μg of nuclear DNA with that of different amounts of a plasmid containing the 3′ end region of the SAPA clone revealed the presence of less than 10 copies of SAPA-related genes in the *T. cruzi* genome (Fig. 1C). Taken together, these results demonstrate that SAPA is encoded by a small family of at least 6 different genes some of which differ in the length of the repeat region (see below).

3.2. *Structure of SAPA gene*

Three independent genomic clones were isolated from a λ gtWES library and characterized by restriction mapping and cross-hybridization studies (Fig. 2A). The location of the open reading frame within clone 28 was determined from its nucleotide sequence which will be presented elsewhere (Pollevick et al., manuscript in preparation). Clones 41, 42 and 28 contained the *Pst*I fragments of 4.8, 4.3 and 2.8 kb, respectively, that were

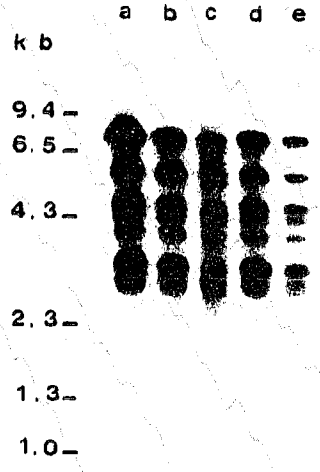

Fig. 3. Sensitivity of SAPA genes to Bal31 digestion. Trypomastigote genomic DNA was treated with Bal31 during increasing period of time: 0 (a), 1 (b), 5 (c), 20 (d) and 30 min (e). The DNA samples were restricted with *Pst*I, blotted and hybridized to the SAPA clone. Molecular weight marker as in Fig. 1.

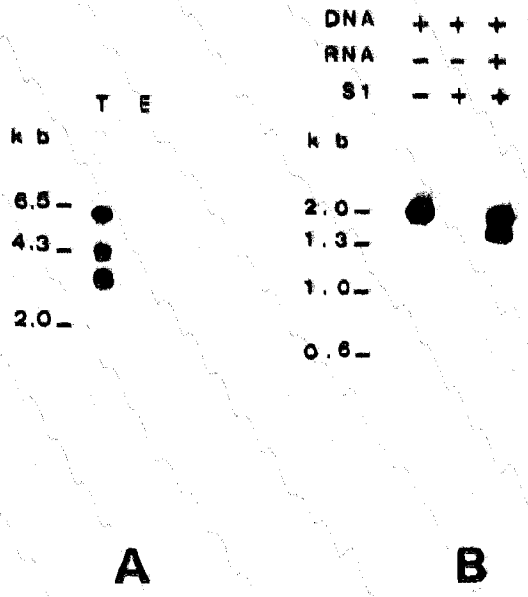

Fig. 4. Transcription of SAPA genes. (A) Northern blot analysis of total trypomastigote (T) and epimastigote (E) RNA. Total RNA was size-fractionated on 1% (w/v) agarose gel, blotted and hybridized to the SAPA clone. The molecular weight marker (*Hin*dIII-restricted λ DNA) is represented on the left of the figure. (B) S1 protection experiment. For detail see section 2. In lane 1, one-tenth of the total amount of the sample was loaded. The molecular weight markers indicated on the left of the figure correspond to *Hin*dIII-digested λ DNA and *Hae*III-restricted Φ X 174 DNA.

detected by Southern blot (Fig. 2A). The restriction map of these clones showed that they correspond to 3 genes that differ mainly in the number of repeat units (Fig. 2A). The DNA sequences upstream and downstream of the repeat region, though presenting some restriction enzyme polymorphisms, seem to be highly conserved as deduced from the analysis of restriction sites (Fig. 2A) and crosshybridizations (not shown). Therefore, it is likely that the variation in length of the repeat region among SAPA genes accounts for their different size. The presence of different number of repeats was extended to other members of the SAPA gene family by means of Southern blotting (Fig. 2B). Exploiting the fact that the conserved *Pvu*II and *Hae*II sites flank the repeat region, we estimated its length in the genomic regions spanning the *Pst*I fragments of 7, 5.5 and 3.7 kb (Fig. 2B). Moreover, of all the endonucleases tested, digestion with *Pvu*II-*Hae*II generated the smallest fragments that hybridized with a repeat probe and which were only sensitive to *Rsa*I that cuts once within each repeat (not shown). The repeat regions of the 5.5- and 3.7-kb fragments are similar in length to those of clone 42 (Fig. 2B). However, the presence of *Pst*I polymorphisms explains the difference in fragment size among them (Fig. 2B). The 7-kb *Pst*I fragment corresponds to the gene bearing the largest repeat region (Fig. 2B). Therefore, 3 out of the 6 SAPA genes described exhibit repeat regions of the same size, which correlates with the detection of 4 hybridization bands with *Pst*I-*Hae*III restricted DNA (see Fig. 1A).

3.3. *Sensitivity to Bal31*

The preferential sensitivity to Bal31 exonuclease of the expressed copy of the VSG genes has provided evidence for its telomeric location [11]. Therefore, we used this approach to study the genomic environment of SAPA genes. All the *Pst*I fragments representing the different members of the SAPA gene family proved to be insensitive to the nuclease treatment under conditions that ensured the digestion of 7–10 kb from each DNA end (Fig. 3). This result indicates that SAPA genes are not located near chromosomal telomeres.

3.4. *Transcription of the SAPA gene family*

To assess the pattern of expression of SAPA genes during the *T. cruzi* life cycle, Northern blots of total trypomastigote and epimastigote RNA were hybridized to the SAPA clone. No hybridization was observed with epimastigotes RNA, whereas transcripts of 6.3, 4.3 and 2.8 kb were detected in the infective form of the parasite (Fig. 4A). Interestingly, the difference in size among the mRNAs was similar to that described for the repeat region of SAPA genes, suggesting that all these genes may be transcribed. To establish whether the RNA transcripts were indeed generated by the expression of different members of the SAPA gene family, or whether they represented processed RNA products of a single gene, S1 protection experiments were carried out. A 2-kb *Pvu*II-*Hae*II fragment encompassing the repeat region of clone 41 was used as probe. If the genes represented by clones 41, 42 and 28 were transcribed, they would give rise to transcripts possessing repeat regions of the same size as those present in the mentioned clones. Hence, protected fragments representing the repeat regions of these genes would be obtained. As expected, 3 protected fragments of 2, 1.4 and 0.6 kb were detected (Fig. 4B) indicating that 3 genes bearing repeat regions of the same size as those of clones 41, 42 and 28 are transcribed. These results strongly support the view that most (or all) SAPA genes are active in the infective form of the parasite. The evidence for the presence of genes bearing repeat regions of 4 different lengths which are actively transcribed provides a plausible explanation for the detection by Western blotting of 4 SAPA-related polypeptides in the supernatant of infected cells and in plasma of infected animals [4].

The results presented herein show that the genomic organization and transcription properties of SAPA gene family are clearly different from those of the multigene family encoding the 85-kDa antigen described by Peterson et al. [6], possibly reflecting a distinct role of these antigens during infection. The 85-kDa antigen could participate in immune evasion like the VSGs of African trypanosomes while SAPA could play a different physiological role. SAPA is expressed in the

acute phase of the disease during which the parasite requires molecules involved in functions other than immune evasion in order to disseminate in the mammalian host. Our results lead to the conclusion that different genetic mechanisms controlling developmental antigenic expression are operating in *T. cruzi*

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