Nucleotide sequence and transcription of a trypomastigote surface antigen gene of *Trypanosoma cruzi*

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(Received 21 September 1990; accepted 27 November 1990)

In previous studies we identified a 500-bp segment of the gene, TSA-1, which encodes an 85-kDa trypomastigote-specific surface antigen of the Peru strain of *Trypanosoma cruzi*. TSA-1 was shown to be located at a telomeric site and to contain a 27-bp tandem repeat unit within the coding region. This repeat unit defines a discrete subset of a multigene family and places the TSA-1 gene within this subset. In this study, we present the complete nucleotide sequence of the TSA-1 gene from the Peru strain. By homology matrix analysis, fragments of two other trypomastigote specific surface antigen genes, pTt34 and SA85-1.1, are shown to have extensive sequence homology with TSA-1 indicating that these genes are members of the same gene family as TSA-1. The TSA-1 subfamily was also found to be active in two other strains of *T. cruzi*, one of which contains multiple telomeric members and one of which contains a single non-telomeric member, suggesting that transcription is not necessarily dependent on the gene being located at a telomeric site. Also, while some of the sequences found in this gene family are present in 2 size classes of poly(A)\(^+\) RNA, others appear to be restricted to only 1 of the 2 RNA classes.

Key words: *Trypanosoma cruzi*; Trypomastigote surface antigen gene

**Introduction**

*Trypanosoma cruzi* is a flagellated parasitic protozoan and the causative agent of Chagas' disease, a serious health hazard throughout much of Central and South America [1]. The parasite has several morphologically different forms during its life cycle, which is divided between the insect vector and the mammalian host. In the insect vector the dividing form of the parasite is the non-invasive epimastigote. Upon migration of the epimastigote to the hindgut of the insect, it transforms to the non-dividing but invasive trypomastigote stage.

In the mammalian host the parasite circulates in the bloodstream as the infective trypomastigote. Upon penetration of the host cell it transforms to the intracellular amastigote, which is the dividing form of the parasite in the mammalian host. Since the trypomastigote is the major invasive form of the parasite in the mammalian host and is exposed to the host defensive mechanisms, much attention has been focused on the surface proteins of this stage of the parasite as potential protective immunogens against infection. Particular attention has been given to surface glycoproteins of 85 kDa, primarily because they constitute the major surface glycoproteins found on the surface of the invasive trypomastigote [2–4] and have been implicated in the
Materials and Methods

Parasite strains and culture. The *T. cruzi* Peru strain was obtained from Stuart M. Krassner, University of California, Irvine. Clonal lines of this strain were established from individual parasites which were isolated by micromanipulation using procedures provided by James Dvorak, National Institutes of Health, Bethesda, MD. Peru clone 3 was utilized for these studies. The cloned *T. cruzi* lines Esmeraldo clone 3 and Silvio X10 clone 1 were obtained from James Dvorak. Growth and maintenance of epimastigotes and tissue-culture derived trypomastigotes of these strains are as described elsewhere.

Nucleic acid isolation, radiolabeling, Southern and Northern transfer, and restriction enzymes. Parasite nuclear DNA, bacterial plasmid DNA and phage λ DNA were isolated as described previously. Agarose gel electrophoresis of DNA, Southern transfer, Northern transfer, prehybridization, hybridization and filter washing were performed as described. DNA restriction fragments were radiolabeled with [α-32P]dNTP using a nick translation kit from Bethesda Research Laboratory (Gaithersburg, MD). Synthetic oligonucleotides were end-labeled using T4 polynucleotide kinase (Boehringer-Mannheim, Indianapolis, IN) and [γ-32P]ATP. All restriction enzymes were purchased from Boehringer-Mannheim and used as recommended.

Isolation of cDNA and genomic clones. A cDNA library constructed in phage λgt10 using trypomastigote poly(A)+ RNA was screened with a 27-nucleotide nt synthetic oligomer representing one unit of the tandem repeat array present in the 85-kDa surface protein gene. Inserts present in phage that showed hybridization were excised, subcloned and characterized by both restriction enzyme mapping and direct nucleotide sequence analysis.

A Peru genomic library was constructed in λDASH (Stratagene, La Jolla, CA) using DNA isolated from culture form trypomastigotes. Genomic DNA was partially digested with endonuclease SauII, size fractionated on a 1% agarose gel, and fragments in the size range 10–20 kb were excised.
Fig. 1. Restriction map and sequencing strategy of the TSA-1 genomic clones Tcg 2 and Tcg 3 and cDNA clones Tcc 1.27 and Tcc 1.22. The hatched boxed regions denote the position of the 27-bp tandem repeat sequence.

and ligated into the SalI site of the vector.

**DNA sequencing.** The strategy for determining the nucleotide sequence of the cDNA and genomic DNA fragments which encode TSA-1 is shown in Fig. 1. DNA sequencing was performed using the dideoxynucleotide chain termination method [17] with 32P-labeled deoxynucleotide triphosphates and T. cruzi-derived DNAs inserted into the Blue-script vector (Stratagene, La Jolla, CA).

**Results**

*Isolation of cDNA and genomic DNA encoding TSA-1.* We previously reported the isolation and partial characterization of 25 recombinant λgt10 phage which contain the 27-bp repeat within the cDNA insert [8]. In order to select cDNAs which would be useful for determining the complete sequence of the 85-kDa trypomastigote specific surface antigen (TSA-1) gene, the nucleotide sequence of the 5’ and 3’ ends of each of the T. cruzi DNA inserts was ascertained. Of the 25 cDNAs examined, two, designated Tcc 1.22 and Tcc 1.27, were chosen for further analysis (Fig. 1). Tcc 1.22 was selected because the 8 bases at its 5’ terminus are identical to those at the 3’ end of the minixon [19], indicating that it likely contains the 5’ most sequences present in the mature TSA-1 mRNA. Tcc 1.22, however, does not contain the 3’ terminus of the mature TSA-1 mRNA. Synthesis of the cDNA insert appears to have initiated within the 27-bp tandem repeat units, since sequences at the 3’ terminus of Tcc 1.22 align perfectly with the 5’ sequences present in Tcg-1, a cloned 500-bp genomic fragment containing the tandem repeat motif of the gene [7]. It is also possible that cDNA synthesis initiated 3’ downstream of the repeat motif and that only partial synthesis of the second strand of the cDNA occurred. To obtain the sequence of the 3’ portion of TSA-1, the nucleotide sequence of the DNA insert in phage Tcc 1.27 was determined. The 5’ end of Tcc 1.27 extensively overlaps with the 3’ end of the insert in Tcc 1.22, and its 3’ end terminates in a poly(A) stretch which is identical in position to 11 other cDNAs which also terminate in a stretch of A residues.

To obtain genomic DNA fragments which contain the TSA-1 gene, a detailed restriction map of the cDNA inserts in Tcc 1.22 and Tcc 1.27 was generated. The results of this analysis revealed the presence of a single SalI restriction site approximately 700 bp upstream of the 3’ end of the insert in Tcc 1.27. The absence of a second SalI site within the cDNA inserts suggested that a genomic SalI DNA fragment should contain approximately 3 kb of the TSA-1 gene as well as additional bases 5’ upstream of the trans-splicing site. This prediction was confirmed by restriction enzyme analysis of total genomic DNA which revealed the presence of a SalI
site approximately 10.5 kb upstream of the SalI site in Tcc 1.27. To clone and isolate the genomic copy of TSA-1, a recombinant library of SalI-digested genomic DNA was constructed in λDASH. Approximately 400000 recombinant phage were obtained and 150000 were screened by hybridization with the 27-nt repeat unit. Four phage showed positive signals upon subsequent rescreening. The T. cruzi DNA inserts in each phage were characterized by restriction enzyme mapping and partial nucleotide sequence analysis. Two of the phage contained single SalI inserts of about 16 kb. Each insert also contained an internal 4.8-kb EcoRI fragment diagnostic of a non-telomeric, non-transcribed member of the subfamily [8]. The remaining 2 phage, designated Tcg-2 and Tcg-3 each contained SalI inserts of 10.5 and 8.0 kb. Restriction mapping of total genomic DNA showed that both fragments lie adjacent within the genome with the 10.5-kb fragment mapping proximal to the telomere. The 10.5-kb SalI fragment also was shown by hybridization analysis to contain sequences homologous to the 27-bp repeat. Therefore, it was excised, subcloned into the plasmid vector Bluescript and subjected to restriction enzyme analysis. As shown in Fig. 1, the restriction enzyme pattern of the 3' region of the 10.5-kb fragment overlaps perfectly with that of the two cDNA inserts, suggesting that it is the genomic site of the TSA-1 gene.

**Sequence analysis.** In order to obtain information on the structure of the TSA-1 gene and the protein which it putatively encodes, the complete nucleotide sequence of the 2 cDNAs and selected regions of the 10.5-kb SalI genomic DNA fragment were determined by the dideoxy chain termination method [17]. Also, the position of 6-bp restriction enzyme recognition sites predicted by either the sequence or restriction mapping was confirmed. The nucleotide sequence of the TSA-1 gene is shown in Fig. 2 and underscored by the predicted amino acid sequence of the protein.

The sequence of the cDNA differed from the genomic DNA only at 15 nucleotides at the 5' terminus (Fig. 2), confirming our supposition that the 2 cDNAs are derived from the same gene. The first 8 nucleotides of the cDNA (i.e., −222 to −215) likely represent the synthetic EcoRI site introduced during construction of the library. The 8 nucleotides immediately following the EcoRI linker are identical to those on the 3' terminus of the miniexon [19], suggesting that the trans-splicing site for miniexon addition is between nucleotides −207 and −206. Consistent with this assignment is the presence of an AG dinucleotide in the genomic DNA immediately 5' of the putative splice junction. The 3' end of the gene is tentatively defined by the presence of A residues at the 3' terminus of the cDNA. Since the genomic region corresponding to this portion of the cDNA has not been cloned, a definitive assignment of the 3' terminus cannot be made. Nevertheless, the comparison of twelve cDNAs which possess A residues at the 3' terminus show no variation in the position of the stretch of A residues, suggesting that the site of post-transcriptional poly(A) addition is at or immediately downstream of the C residue at position 3495. Interestingly, the sequence AA-TAAA at position 3459 is identical to the consensus sequence AAATAAA that precedes the polyadenylation site of most eukaryotic mRNAs by 12–40 nucleotides. However, since this sequence has not yet been observed in other T. cruzi genes [20], its presence here may be fortuitous and merely reflect the high concentration of A and T residues in the 3' untranslated region of T. cruzi mRNA.
TABLE I
Comparison of sequences 5' of potential translation initiation sites for TSA-1 with consensus initiation sequences of eukaryotic protein coding genes and the initiation sequence for other T. cruzi protein coding genes

| Consensus sequence | TSA-1 First ATG | Second ATG | Third ATG | Hsp-85 | IF8 | pTC-FUS1 |
|--------------------|----------------|------------|-----------|--------|-----|---------|
|                     | C C A/G C C    | T T T A T | C C A A C | C A A A G | A C G A G | A A A C C |
|                     | ATG            | ATG        | ATG       | ATG    | ATG | ATG     |

*The rules used for assignment of consensus are described elsewhere [23].
*bNucleotides −116 to −109 in Fig. 2.
*cNucleotides −5 to 3 in Fig. 2.
*dNucleotides 47 to 54 in Fig. 2.
*eThe ATG initiation codon and the 5 nt immediately 5' to this initiation site in the T. cruzi 85-kDa heat shock protein, Hsp-85 [13].
*fThe ATG initiation codon and the 5 nt immediately 5' to this initiation site in the T. cruzi Ca²⁺ binding protein gene, IF8 [21].
*gThe ATG initiation codon and the 5 nt immediately 5' to this initiation site in the T. cruzi ubiquitin gene, pTc-FUS [22].

Identification of the protein coding region. Translation of the TSA-1 DNA sequence reveals the presence of a single long open reading frame. Within this reading frame the first ATG is 112 bp downstream of the 5' end at position −111 (Fig. 2). Translation starting at this site would end at nt position 2507 and yield an 872-amino acid (aa) protein of 94513 Da. A second and third ATG triplet are found in the TSA-1 transcript at nt positions 1 and 52, and are in the same reading frame as the first ATG. Translation starting at the second or third ATG would result in proteins of 835 aa and 818 aa, respectively, with predicted Mr's of 90429 and 88430. Each of the predicted Mr's are larger than the apparent Mr of 85000 previously reported [4-7,9,10], suggesting that processing of the primary translation product might be occurring. As discussed below, analysis of the putative translation product is in keeping with this suggestion. Examination of the 5' sequences flanking the three potential translation start sites shows that only those sequences upstream of the second ATG strongly match the generalized eukaryotic consensus sequence proposed by Kozak [23] (Table I). Therefore, we have tentatively assigned the second ATG triplet as the start codon.

Nucleotide homology between TSA-1, pTt34 and SA85-1.1. Two DNA fragments, pTt34 [9] and SA85-1.1 [10], which represent parts of genes which encode 85-kDa trypomastigote specific surface antigens, have been cloned and characterized. A homology matrix analysis of nucleotide identities between TSA-1 and both pTt34 and SA85-1.1 is shown in Fig. 3. Position homology between the 2 sequences is scored by placement of a symbol if 21 out of 30 nt (i.e., 70%) showed identities in a sequential scan of the DNA sequence. In the comparison
with pTt34, it is clear that considerable homology exists with TSA-1 in the predicted coding region in the 5' end of the gene. Most of the scored homologies lie on a single continuous linear axis with only a few regions being identified elsewhere within the sequence. The high degree of homology observed in this alignment (i.e., about 68%) indicates that this portion of the coding region of the 2 genes has been maintained in length with few, if any large deletions or gene rearrangements.

In the comparison with SA85-1.1, most of the homology exists within the extreme 3' end of the predicted coding region and the contiguous noncoding region of TSA85-1 with only limited homology being evident elsewhere within the region. The 2 regions of high homology observed within the predicted coding region of TSA-1 occur within regions of 43 bp (nt 1901–1944) and 84 bp (nt 2424–2508) which show 91% and 70% homology, respectively. Not all of the scored homologies lie on a linear array, suggesting that these regions of the 2 genes have been conserved but some rearrangements have occurred in the form of deletions and/or insertions. Similar results were obtained when the nucleotide sequence of TSA-1 was compared with the sequence of 2 other members of the SA85-1 gene family, SA85-1.2 and SA85-1.3 (data not shown).

**Strain-specific expression of the TSA-1 gene subfamily.** Our previous results with the Peru strain have shown that the 27-bp repeat unit in the TSA-1 gene defines a subfamily of a larger 85-kDa gene family. To determine whether the repeat motif which defines this subfamily was present in strains of T. cruzi other than Peru, a Southern blot containing total genomic DNA from the Peru, Esmeraldo and Silvio X10 strains was hybridized with the 27-nt repeat unit (Fig. 4). Hybridization was observed to 4 EcoRI restriction fragments in both the Peru and Esmeraldo DNA and to a single EcoRI fragment in Silvio X10 DNA, indicating that the TSA-1 subfamily is present in all 3 strains of the parasite.

To determine whether members of the subfamily in the Esmeraldo and Silvio X10 strains were also transcribed, the 27-nt repeat unit was hybridized to Northern blots containing poly(A)+ RNA from each of the strains. As shown in Fig. 5A, the 27-nt repeat unit hybridized to RNA of 3.7 kb from both the Peru and Esmeraldo strains and to RNA size 3.4 kb from the Silvio X10 strain, suggesting that at least 1
member of this subfamily is being expressed in each of the 3 strains.

The 85-kDa gene family is expressed in more than 1 size class of poly(A)^+ RNA. Our previous studies suggested that in the Peru strain sequences homologous to members of the TSA-1 gene family may be present in two size classes of poly(A)^+ RNA, of average length 3.7 and 3.4 kb [8]. The repeat motif, however, was observed only in the 3.7-kb class of RNA. In view of the current observation that in the Silvio X10 strain the repeat motif is present only in the 3.4-kb class of RNA, and the fact that our previous studies included hybridization data using only a limited region of the TSA-1 gene (i.e., nucleotides 1852–2342 in Fig. 2), we have extended these studies to include sequences 5′ upstream and 3′ downstream of this 490 bp region. As shown in Fig. 5B, the full length transcript of TSA-1 hybridized to 2 differently sized RNAs in each of the 3 strains. In the Peru and Esmeraldo strains, TSA-1 shows a strong hybridization signal with poly(A)^+ RNA of 3.7 kb and a less intense signal with poly(A)^+ RNA of 3.4 kb. However, in contrast to the hybridization profile observed with the Peru and Esmeraldo RNAs, the length of the larger RNA species in Silvio X10 (i.e., 3.6 kb) is slightly less than observed in the other 2 strains, and the hybridization signal of the 3.4-kb RNA species is considerably more intense than that observed with the 3.6-kb RNA.

To further define which regions of TSA-1 are represented in the two poly(A)^+ RNA size classes, selected regions of the TSA-1 gene were hybridized to Northern blots containing poly(A)^+ RNA from the 3 strains. As shown in Fig. 5C, the 1.8-kb BamHI/EcoRI fragment which contains most of the 5′ end of the gene (i.e., nucleotides −51 to 1857 in Fig. 2) hybridized to both RNA classes in all 3 strains. As previously observed with the full length TSA-1 gene, the intensity of the hybridization signal was greatest with the 3.7-kb RNA species from Peru and Esmeraldo strains and the 3.4-kb RNA species from the Silvio X10 strain. As previously observed with the full length TSA-1 gene, the intensity of the hybridization signal was greatest with the 3.7-kb RNA species from Peru and Esmeraldo strains and the 3.4-kb RNA species from the Silvio X10 strain. In contrast, the SalI/EcoRI fragment which contains 744 bp of the 3′ end of the gene (i.e., nucleotides 2761–3505 in Fig. 2) hybridized only to the 3.6 and 3.7 kb RNA (Fig. 5D). These results and those reported previously [8] indicate that sequences found 5′ of the repeat motif in the TSA-1 transcript share homology with 2 different size classes of RNA, while both the repeat motif and sequences 3′ of the repeat are present only in one size class of RNA.

BAL 31 nuclease sensitivity of the TSA-1 homologues. Of the 4 EcoRI restriction fragments which contain the 27-bp repeat motif in the Peru strain, 1 has been shown to be telomeric in location and has been implicated as the site of transcription of the TSA-1 RNA [8]. The remaining 3 fragments are not located near a telomere and appear to be either transcriptionally silent or transcribed at a very low level. We have examined the possibility that 1 or more of the EcoRI fragments containing the repeat motif in the Esmeraldo strain, and the single EcoRI fragment in the Silvio X10 strain, are telomeric by the technique of preferential sensitivity to digestion with BAL 31 nuclease.
As shown in Fig. 6, 3 of the 4 EcoRI fragments in the Esmeraldo strain are preferentially sensitive to BAL 31 digestion, which suggests that these three members of the subfamily are telomeric in location. In contrast, the single EcoRI fragment in the Silvio X10 strain is not preferentially sensitive to BAL 31 nuclease, which suggests that the subfamily in Silvio X10 has no telomeric member.

**Discussion**

Extensive searches of data banks and the literature have failed to reveal any strong homology between TSA-1 and other genes or proteins whose biological function is known. Therefore, we do not have any substantial clues as to the function of TSA-1. However, the search did reveal that TSA-1 has extensive sequence homology with two other cDNA fragments which have been shown to encode 85 kDa trypomastigote specific surface antigens, pTt34 and SA85-1.1 [9,10]. Although SA85-1.1 was believed to be a member of a gene family other than that containing either pTt34 or TSA-1 [10], it now seems likely that TSA-1, pTt34 and SA85-1.1 are members of the same multigene family. Therefore, it is quite possible that the differences observed in the physical properties of the 85-kDa surface antigen [4-6] are due to diversity within this single gene family.

Although the functions of the major 85-kDa surface glycoproteins of T. cruzi are not known, biological and physical properties of these glycoproteins have been reported [2-6]. It is therefore worthwhile to determine how certain features of the predicted protein sequence of TSA-1 relate with these properties. The N-terminus contains a hydrophobic region which is compatible with an N-terminal signal peptide [25], with a reasonable candidate signal peptide processing site being present at residue 29 [26,27]. The protein contains no hydrophobic region at the COOH-terminus which would serve as a transmembrane domain, but it does possess a hydrophobic stretch of amino acids at the COOH-terminus that could serve as a processing site for a phosphatidylinositol linkage [28]. As noted previously [7], the antigen contains a 9-amino-acid repetitive peptide sequence proximal to the COOH-terminus of the protein. Of the 9 residues in the repeat unit, 4 are charged, making this region of the protein ostensibly hydrophilic. Accordingly, computer analysis shows that the region of the protein containing the repeat motif is extensively hydrophilic and would likely represent an area of high antigenicity. Consistent with this interpretation, synthetic oligopeptides containing 2 repeat units arranged in a head-to-tail tandem array are recognized by antibodies from Chagasic patients and mice infected with T. cruzi (Wrightsman and Manning, unpublished). Finally, the protein contains several potential sites for attachment of N-linked glycosyl moieties. Each of these properties are consistent with the observation that the 85-kDa protein is a highly antigenic surface glycoprotein.

**Transcription of TSA-1.** Our previous results with the Peru strain have shown that of the four members of the TSA-1 gene family which are defined by the presence of the 27-bp repeat motif, only the single telomeric member appears to be transcribed into poly(A)^+ RNA [8]. While this also appears to be true for the Esmeraldo strain (Fouts and Manning, unpublished), it is clearly not the case for the member of the subfamily found in the Silvio X10 strain. BAL 31 analysis of Silvio X10 genomic DNA reveals the presence of a single member of the subfamily which is located at a non-telomeric site. Northern blot analysis indicates that this member is transcribed into an abundant poly(A)^+ RNA, thus indicating that members of this subfamily need not necessarily be located at a telomeric site in order to be transcriptionally active.

It is clear that at least two different poly(A)^+ RNA size classes share sequence homology with TSA-1 (Fig. 5) and that the presence of the 27-bp repeat unit in these 2 size classes of RNA differs among strains. In the Peru and Esmeraldo strains, the sequences within the coding region of the TSA-1 gene which are 5' upstream of the 27-bp repeat are represented in both the 3.7- and 3.4-kb RNAs, while the repetitive sequence and those sequences downstream of the repeat share homology only with the 3.7-kb poly(A)^+ RNA. Conversely, in the Silvio X10 strain the repeat motif is present only in the smaller 3.4-kb RNA while those sequences 3' of the repeat motif in TSA-1 are present only in the larger 3.6-kb RNA. It is very clear, therefore, that the sequences present 3' downstream of the repeat motif in the Peru and Esmeraldo gene(s) are not pre-
sent in the Silvio X10 transcript.

Although we do not yet understand the relationship between the two classes of RNA which encode this gene family, 2 possibilities present themselves with regards to the origin of the transcripts. One is that the 3.4-kb RNA is a processed form of the 3.7-kb transcript. Alternatively, the 2 RNA classes could originate from different genes within the family. Although we cannot formally exclude the possibility of processing, we favor the suggestion that the 2 classes of RNA arise independently by transcription of different genes. This is based on the observation that although the 2 RNA classes differ by only 300 bp in the Peru and Esmeraldo strains, the 3.7-kb RNA shares homology with sequences found throughout the 1.5-kb 3' terminus of TSA-1, while the 3.4-kb RNA shows no homology with this 1.5-kb region of the gene. It is difficult to imagine, therefore, how the 3.7-kb transcript can be processed to yield the 3.4-kb RNA. The question also arises as to whether the different size RNAs might provide alternate properties to the protein. Several feasible possibilities present themselves and are currently being investigated.

Acknowledgements

We thank Barbara Granger for assistance in maintenance and growth of parasite cultures, Stuart Kahn and Harvey Eisen for thoughtful suggestions and sharing unpublished data, and Jim Massey for technical assistance. This work was supported by Public Health Service grant AI18873 from the National Institutes of Health.

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