Trypanosoma cruzi: identification and characterization of a novel ribosomal protein L27 (TcrL27) that cross-reacts with an affinity-purified anti-Sm antibody

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SUMMARY

Small nuclear ribonucleoproteins (snRNPs) are involved in trans-splicing processing of pre-mRNA in *Trypanosoma cruzi*. To clone *T. cruzi* snRNPs we screened an epimastigote cDNA library with a purified antibody raised against the Sm-binding site of a yeast sequence. A clone was obtained containing a 507 bp-insert with an ORF of 399 bp and coding for a protein of 133 amino acids. Sequence analysis revealed high identity with the L27 ribosomal proteins from different species including: *Canis familiaris*, *Homo sapiens*, *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*. This protein has not been previously described in the literature and seems to be a new ribosomal protein in *T. cruzi* and was given the code TcrL27. To express this recombinant *T. cruzi* L27 ribosomal protein in *E. coli*, the insert was subcloned into the pET32a vector and a 26 kDa recombinant protein was purified. Immunoblotting studies demonstrated that this purified recombinant protein was recognized by the same anti-Sm serum used in the library screening as well as by chagasic and systemic lupus erythemathosus (SLE) sera. Our results suggest that the *T. cruzi* L27 ribosomal protein may be involved in autoimmunity of Chagas disease.

Key words: *Trypanosoma cruzi*, recombinant protein, immunoscreening, Western blot, Southern blot, TcrL27.

INTRODUCTION

Chagas disease or American trypanosomiasis is a zoonosis caused by the flagellate protozoan *Trypanosoma cruzi*, which is transmitted by haematophagous insects belonging to the sub-family Triatominae. This New World disease extends from Mexico to Argentina. Estimates indicate that there are 16–18 million people infected in Latin America (WHO, 1995; Moncayo, 1999).

The aim of this work was to identify candidate genes whose products may be involved in autoimmune responses during infection with *T. cruzi*. Several autoimmune responses have been detected in Chagas patients (Van Voorhis *et al*. 1993). Some of these responses have been explained by the mimicry between host and parasite proteins which can induce autoantibodies by molecular cross-reaction with host structures from cardiac or nerve tissues (Miatello & Fiorotto, 1989). Since its aethiology is known, Chagas disease is an important model for the study of autoimmunity. This model permits the study of how autoantibody production is initiated, including those autoantibodies not related, thus far, to Chagas disease (Cicarelli *et al*. 1998).

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During the chronic phase of a *T. cruzi* infection, there is irreversible and progressive damage of some organs, especially the heart, oesophagus, and colon and is responsible for the cardiac or digestive forms of the disease (Tanowitz *et al*. 1992). It is common to find inflammatory lymphocytes and macrophages infiltrating into the heart and it is hypothesized that an autoimmune mechanism contributes to the physiopathology of Chagas disease (Van Voorhis *et al*. 1993).

Sm epitopes, Sm-binding site (Sm means Smith, patient from whom this antigen was detected), are usually recognized by sera from subgroups of patients with SLE. Anti-Sm antibodies have been shown to recognize principally 3 nuclear polypeptides of molecular weight 28 000/29 000 (B/B′) and 16 000 (D) which associate with U1, U2, U4, U5, and U6 RNA. Those RNAs are present in all snRNPs (small nuclear ribonucleoproteins) that are part of the spliceosome which shares a common structural region, named the Sm-binding site or domain A, consisting of a single-strand region rich in uridine which binds to one or more common snRNPs (Seraphin, 1995; Billings & Hoch, 1983; Conner *et al*. 1982).

Bach *et al*. (1998) investigated the presence of autoantibodies in sera from patients with Chagas disease against the epitopes of the RNPs and showed that 61% of chagasic sera recognized this Sm epitope. Using sera from selected patients with Chagas

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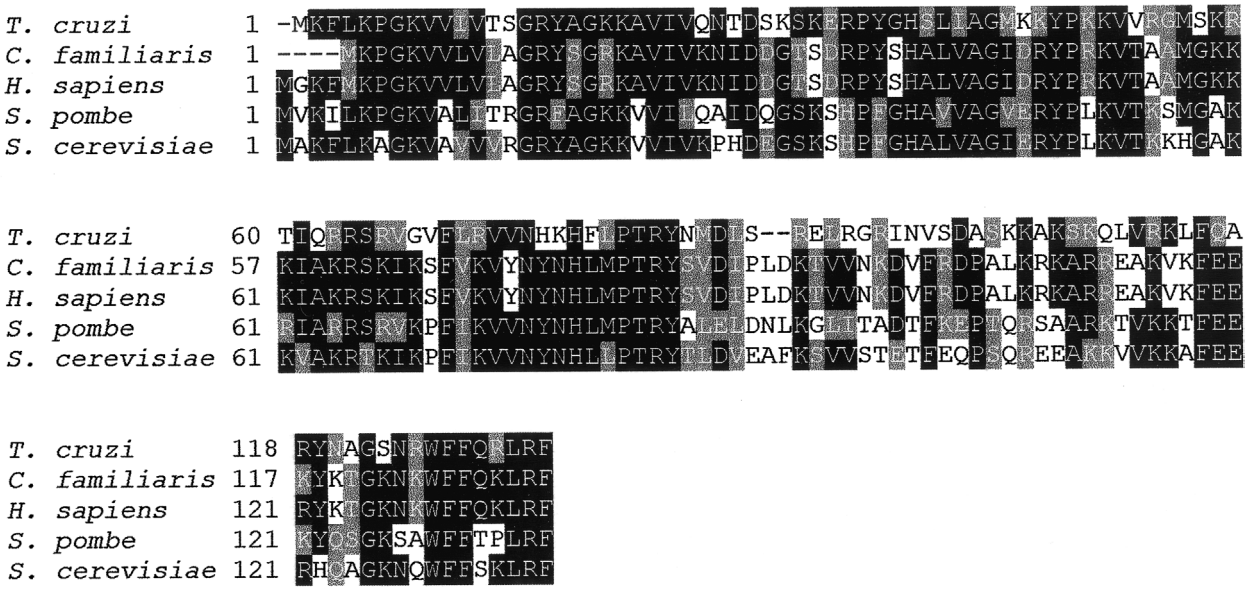

Fig. 1. Multiple alignment of the deduced amino acid sequence of TcrL27 and 4 ribosomal L27 protein sequences. The black boxes indicate amino acid identity and the grey boxes represent conservative substitutions. Accession number AF176558.

disease and *T. cruzi* purified RNPs in ELISA, the authors found that these sera contained antibodies that react to human and to *T. cruzi* RNPs. The simultaneous presence of the autoantibodies against both human and *T. cruzi* RNPs could suggest an autoimmune response to both antigens. Using purified human UsnRNPs we detected anti-human UsnRNPs antibodies in sera from patients with Chagas disease. The antibodies were also detected using peptide-ELISA containing the Sm-motif 1 domain, showing that 61% (31/51) of the Chagas sera contained antibodies against the Sm-motif 1 (Cicarelli *et al*. 1998).

MATERIALS AND METHODS

Immunoscreening of cDNA library

A *Trypanosoma cruzi* (Y strain – epimastigote forms) cDNA library constructed in Uni-ZAP (Stratagene) was generously provided by Dr Santuza Teixeira (Universidade de Brasilia-DF) and screened with affinity-purified rabbit IgG anti-Sm (dilution 1:50 in PBS + Tween 20). This antibody was obtained from rabbits immunized against the Sm region and later purified using an affinity column with Sm peptide and was provided by Dr Montserrat Bach-Elias, CID-CSIC, Barcelona, Spain. The reaction was developed using anti-rabbit IgG–peroxidase conjugate diluted 1:500 in the same buffer, and revealed with DAB (diaminobenzidine, Gibco/BRL) and H_2O_2. The cloned plasmid pBluescript was digested with the restriction enzymes *Eco*RI and *Xho*I (10 U/μl–Gibco/BRL) to release the insert that was sequenced in both directions using T3 and T4 primers by the Sequencing Services at CSIC, Barcelona, Spain.

Southern blot analysis

Isolation of genomic DNA from epimastigote forms of 3 different *T. cruzi* strains (Y, Bolivia and NCS) was carried out using the Wizard Genomic kit (Promega). Genomic DNAs were digested separately with *Hin*dIII and *Pst*I restriction enzymes, precipitated and resolved on a 0·8% agarose gel and transferred to the nylon membrane. TcrL27 nucleotide sequence showed no restriction sites for these enzymes. The membrane was pre-hybridized at 42 °C (hybridization solution – Gibco/BRL) for at least 4 h and hybridized overnight with a biotin-labelled probe (using a Bioprime kit – Gibco/BRL). After washing twice for 10 min each at room temperature with 1× SSC (3 M NaCl, 0·3 M sodium citrate), 0·1% SDS (sodium dodecyl sulfate); 2×10 min at RT with 0·1× SSC, 0·1% SDS, and 2×15 min at 65 °C with 0·1× SSC, 0·1% SDS, and the probe detection was done using Photogene kit (Gibco-BRL). The membrane was then exposed to the X-ray film for 30 min.

Expression of the recombinant protein and Western blot analysis

The recombinant protein was expressed as a fusion protein using the pET32a expression system; this vector allows the addition of 6 histidine residues at the C-terminus. The insert was cloned in frame at the *Eco*RI and *Xho*I restriction sites and the recombinant plasmid was used to transform BL21pLys competent cells. Bacterial transformants were used to inoculate LB/ampicilin/chloranphenicol medium. After overnight bacterial culture at 37 °C, the cells were subcultured and induced with 10 mM IPTG

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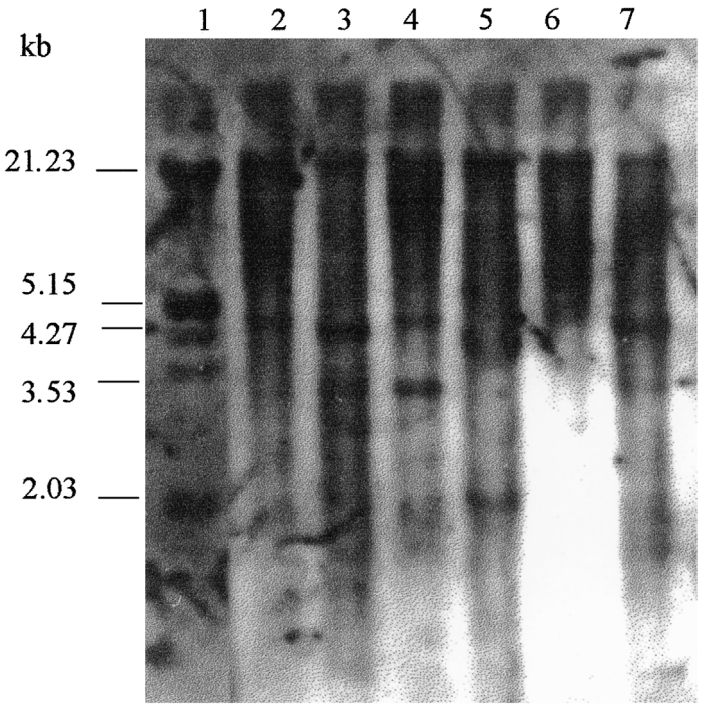

Fig. 2. Southern blot analysis. Total genomic DNA was prepared from 3 different strains and digested with *Hin*dIII (lanes 2, Y; 4, Bolivia; 6, SO strains) and *Pst*I (lanes 3, 5 and 7, Y, Bolivia and SO strains, respectively). Lane 1, biotinylated M_r markers (Gibco/BRL). This analysis used biotinylated-TcrL27 clone as a probe.

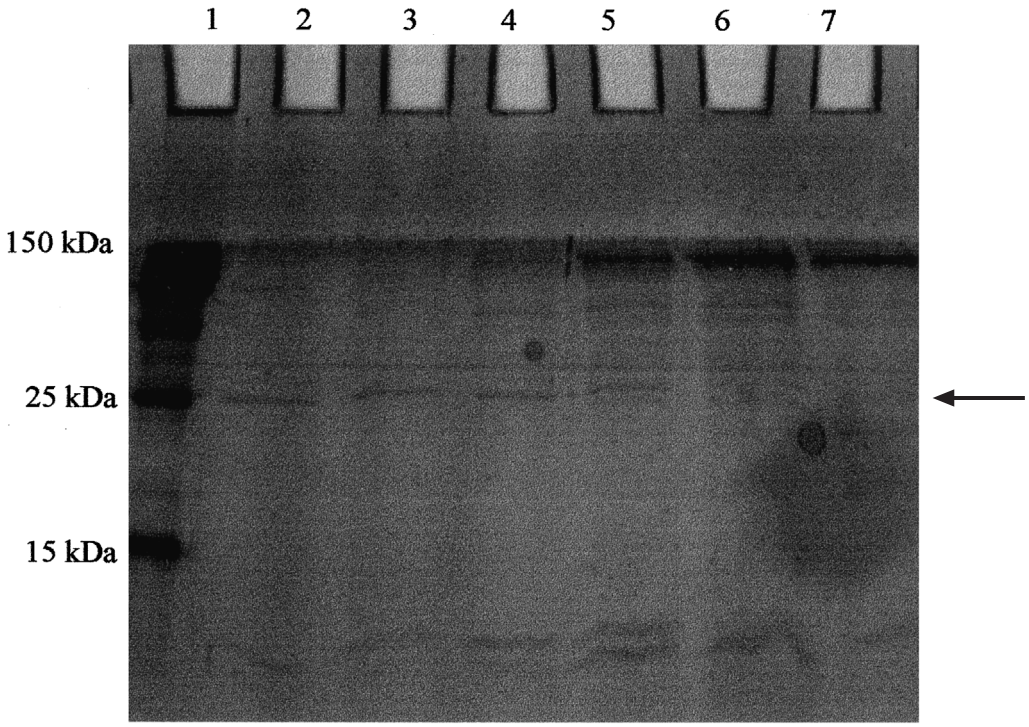

Fig. 3. SDS–PAGE analysis on 15% gels of TcrL27-recombinant protein (26 kDa). Lanes 1, M_r markers (Gibco/BRL); 2–7, eluates collected from affinity-column His-bind. The gel was stained with Coomassie Brilliant Blue. Molecular weights in kDa are indicated. The arrow shows the semi-purified protein.

(isopropylthio-β-D-galactoside, Gibco/BRL) for 2·5 h at the same temperature. Semi-purification of the recombinant protein was performed using an affinity-column (His-bind resin, Novagen), which allows rapid purification of the histidine-tagged proteins. Eluates were carried out using 1 M imidazole, 0·5 M NaCl, 20 mM Tris–HCl, pH 7·0, in different tubes and analysed in a 15% gel by SDS–PAGE (Laemmli, 1970). The recombinant protein was also transferred to a membrane for Western Blot analysis.

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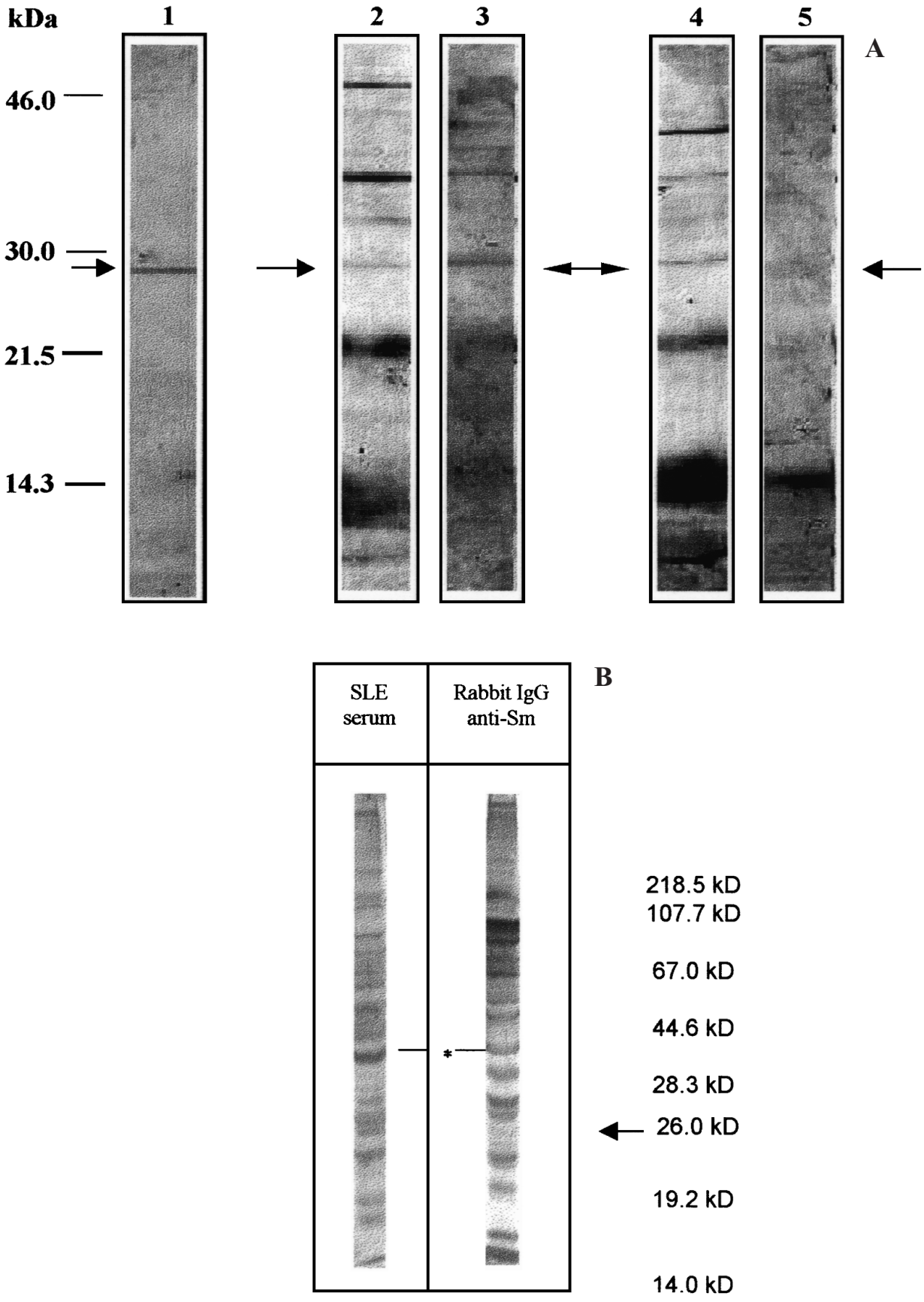

Fig. 4. Western blotting analysis. (A) TcrL27 recombinant protein as antigen using different antibodies revealed with Protein A–peroxidase conjugate and DAB + H_2O_2. Lanes 1, rabbit IgG anti-Sm; 2 and 3, chagasic sera; 4, SLE serum; 5, NHS (normal human serum). The arrow shows the position of TcrL27 recombinant protein (26 kDa).

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The membrane was cut into strips, which were blocked in a 3% low fat milk solution and then incubated with the following sera: rabbit IgG anti-yeast Sm (the same antibody as was used in the library screening), 2 different sera from patients with Chagas disease, 1 from a patient with SLE and 1 normal human serum. After incubation with the sera, the strips were washed and incubated with Protein A–peroxidase conjugate (Sigma). The reactions were developed using DAB and H_2O_2 as substrate solution.

For Western blotting using proteins from *T. cruzi* nuclear extract of epimastigote forms (10^9 cells), the parasite nuclear extract was prepared as described by Dignam *et al.* (1983). A 50 μl sample of nuclear extract was extracted by phenol/chloroform (PCA) and the aqueous phase removed. To the PCA phase 500 μl of acetone were added at -20 °C for 30 min to precipitate the proteins, followed by centrifugation at 21 000 ***g***, for 15 min at 4 °C and the pellet washed with 70% ethanol. The pellet was dried, resuspended in urea dye, electrophoresed in a 10% urea–acrylamide gel (denaturing and preparative gel) and transferred to nitrocellulose membrane. After transfer, the membrane was cut into strips to probe with SLE serum and rabbit IgG anti-Sm. The blots were revealed as already described.

RESULTS

The cloning of the new TcrL27 gene

A *T. cruzi* cDNA library was screened using an affinity-purified IgG anti-yeast Sm obtained by immunization of rabbit against Sm-binding proteins. After the third immunoscreening, the isolated clone was excised '*in vivo*' from the Uni-Zap phage. The entire cDNA sequence of 507 bp showed an open reading frame of 399 bp coding for recombinant *T. cruzi* L27, TcrL27, a new deduced protein with 133 amino acids and a predicted molecular weight of 14 kDa. The complete amino acid sequence was deposited in the GenBank under the Accession Number: AF176558. The deduced amino acid sequence was compared to other L27 ribosomal proteins using the Clustal W program (Fig. 1) and showed approximately 50% identity and 70% similarity with the ribosomal proteins named L27 from different organisms, such as, *Canis familiaris*, *Homo sapiens*, *Schizosaccharomyces pombe* and *Sacchromyces cerevisiae*.

Southern blot analysis using biotin-labelled clone

Fig. 2 shows the results of hybridization with TcrL27 clone as a probe. The presence of multiple bands (even at high stringency wash) suggests that TcrL27 is present as a multiple-copy gene. The genomic DNA samples from different *T. cruzi* strains digested with *Hin*dIII and *Pst*I showed a particular hybridization pattern specific for each strain, which could indicate a genetic polymorphism at this gene locus in different parasite strains. Indeed these results are similar to those found by Elhag & Bourque (1992) and Gallagher, McClean & Malik (1994) studying L27 from *Nicotiana tabacum* and *Homo sapiens*, respectively.

Expression of TcrL27-recombinant protein

Samples of 20 μl of 1 ml-eluates of TcrL27 recombinant protein were electrophoresed on 15% SDS–PAGE and the results are shown in Fig. 3, lanes 2–7. A band at approximately 26 kDa (shown by an arrow) corresponds to the recombinant protein and was eluted without contaminants in tubes 1 and 2.

Western blot analysis of the strips containing the TcrL27-recombinant protein as antigen, incubated with different antibodies, is shown in Fig. 4. The results showed that both chagasic and SLE serum recognized this ribosomal protein (lanes 2–3 and 4, respectively) as well as the rabbit IgG anti-Sm (lane 1). When proteins obtained from *T. cruzi* (Y strain) nuclear extract were used as antigen in Western blot probed with the same IgG anti-Sm or the SLE serum, a 26 kDa protein could also be shown (assigned by *), even when other bands were present.

DISCUSSION

The aim of this work was to clone genes related to *T. cruzi* UsnRNPs, using immunoscreening of an epimastigote cDNA library with an anti-Sm affinity-purified antibody. Unexpectedly, a clone coding for the ribosomal protein L27 in *T. cruzi* was obtained and named TcrL27; this was probably due to the presence of an epitope cross-reactive with the Sm portion present in small nuclear ribonucleoproteins. Expression of the TcrL27-gene in *E. coli* BL21pLysS strain made it possible to prepare and semi-purify the recombinant protein in a His-bind affinity-column and analyse it by Western blot using selected antibodies. The results of immunoblotting demonstrated that the TcrL27 recombinant protein (26 kDa) can be detected either by the anti-Sm antibody, used in the library immunoscreening, or by SLE serum. A protein with the same molecular weight could be shown in nuclear extracts of *T. cruzi* probed with the same antibodies in immunoblotting, confirming the cross-reactivity among these epitopes and the 26 kDa TcrL27.

(B) Proteins of *Trypanosoma cruzi* (Y strain) nuclear extract as antigen probed with SLE serum and rabbit IgG anti-Sm antibody (the same used on library immunoscreening), respectively, showing a similar 26 kDa band (*). Molecular weights are indicated.

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The amino acid sequence of TcrL27 deduced from the nucleotide sequences, when compared with published sequences in the GenBank, revealed significant identify with the L27 proteins from various organisms, demonstrating that these ribosomal proteins are well-conserved among eukaryotes. These proteins also maintain a high degree of homology in terms of amino acid sequence as demonstrated by Coulter & Hide (1996) in the ribosomal protein L18 from *Trypanosoma brucei*, where they obtained 46% and 43% identity with human and yeast sequences, respectively.

An homologous gene, which encodes for the ribosomal 60S protein L27a, was also cloned and characterized from *T. brucei*. Although the amino acid sequence gave 45–58% identity with other L27a (L29) homologues. 'Northern blot analysis of RNA from 3 *T. brucei* life-cycle stages shows that mRNA levels are 2-fold higher in procyclic than in early or late bloodstream stages. This infers that this highly conserved ribosomal protein may play an important role in translational regulation through the life-cycle of trypanosomes' (Brown & Williams, 1999).

We note here that the nucleotide sequence of *T. cruzi* cDNA, which encodes for this new TcrL27 protein, does not have *Hin*dIII or *Pst*I restriction sites, as shown by Southern blot analysis. The results suggest that at least in 3 strains of *T. cruzi* the TcrL27 gene is present as multiple copies.

Using the software program of Biological Information Resource (University of Washington, USA) an approximate calculation of the pI (isoelectric point) for this TcrL27-recombinant protein was 11·78 and is in accordance with the basic feature found in the L27 ribosomal protein from other organisms, as well as, mouse pI 10·5 (Tanaka *et al.* 1988), potato (*Solanum tuberosum L.*) pI 11·36 (Taylor & Davies, 1994), and green algae (*Chlamydobotrys stellata*) pI 10·4 (Wolf *et al.* 1993).

Cross-reactions between ribosomal proteins and UsnRNPs were demonstrated by Nojima *et al.* (1989) using a different approach. Anti-Sm auto-antibodies recognized not only the Sm antigen but also a ribosomal acid protein different from P proteins, identified by them as RP21 protein. Caponi *et al.* (1998) observed that a certain amount of antibody against P-ribosomal human protein, obtained from sera of patients with SLE, could also react with recombinant proteins D and/or B/B′ of the Sm complex. All together, these results present strong evidence suggesting the presence of epitopes in ribosomal proteins and those epitopes could show cross-reactivity and also be recognized by the anti-Sm auto-antibodies.

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REFERENCES

BACH, M., BAHIA, D., TEIXEIRA, D. C. & CICARELLI, R. M. B. (1998). Presence of autoantibodies against small nuclear ribonucleoprotein epitopes in Chagas patients' sera. *Parasitology Research* **84**, 796–799.

BILLINGS, P. B. & HOCH, S. O. (1983). Isolation of intact Sm/RNP antigens from rabbit thymus. *Journal of Immunology* **131**, 347–351.

BROWN, S. V. & WILLIAMS, N. (1999). Analysis of the 60S ribosomal protein L27a (L29) gene of *Trypanosoma brucei*. *International Journal for Parasitology* **29**, 731–736.

CAPONI, L., BOMBARDIERI, S. & MIGLIORINI, P. (1998). Anti-ribosomal antibodies bind the Sm proteins D and B/B′. *Clinical and Experimental Immunology* **112**, 139–143.

CICARELLI, R. M. B., CASTRO, L. I. R., MARTINS, E. C., CEGATTI, M. B., BAHIA, D. & BACH-ELIAS, M. (1998). Spliceosomal factors are recognized by Chagasic sera. *International Congress of Immunology* **10**, 1141–1145.

CONNER, G. E., NELSON, D., WISNIEWOLSKI, R., LAHITA, R. G., BLOBEL, G. & KUNKE, H. G. (1982). Protein antigens of the RNA-protein complexes detected by anti-Sm and anti-RNP antibodies found in serum of patients with systemic lupus erythematosus and related disorders. *Journal of Experimental Medicine* **156**, 1475–1485.

COULTER, L. J. & HIDE, G. (1996). The cloning and sequencing of a ribosomal L18 protein from an evolutionary divergent eukaryote, *Trypanosoma brucei*. *Biochimica et Biophysica Acta* **1309**, 69–72.

DIGNAM, J. D., LEBOVITZ, R. M. & ROEDER, R. G. (1983). Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Research* **11**, 1475–1489.

ELHAG, G. A. & BOURQUE, D. P. (1992). Nuclear-encoded chloroplast ribosomal protein L27 of *Nicotiana tabacum*: cDNA sequence and analysis of mRNA and genes. *Biochemistry* **31**, 6856–6864.

GALLAGHER, R. A., McCLEAN, P. M. & MALIK, A. N. (1994). Cloning and nucleotide sequence of a full length cDNA encoding ribosomal protein L27 from human fetal kidney. *Biochimica et Biophysic Acta* **1217**, 329–322.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

MIATELLO, C. S. & FIOROTTO, E. R. (1989). Ribosomal antibody response in rabbits and mice infected with *Trypanosoma cruzi*. *Revista Argentina de Microbiologia* **69**, 413–416.

MONCAYO, A. (1999). Progress towards interruption of transmission of Chagas disease. *Memorias do Instituto Oswaldo Cruz*. **94** (Suppl. 1), 401–404.

NOJIMA, Y., MINOTA, S., YAMADA, A. & TAKAKU, F. (1989). Identification of an acidic ribosomal protein reactive with anti-Sm autoantibody. *Journal of Immunology* **143**, 1915–1920.

SERAPHIN, B. (1995). Sm and Sm-like proteins belong to a large family: identification of proteins of the U6 as well as the U1, U2, U4 and U5 snRNPs. *EMBO Journal* **14**, 2089–2098.

TANAKA, T., KUWANO, Y., ISHIKAWA, K. & OGATA, K. (1988). Nucleotide sequence of cloned cDNA specific for rat ribosomal protein L27. *European Journal of Biochemistry* **173**, 53–56.

TANOWITZ, H. B., KIRCHHOFF, L. V., SIMON, D., MORRIS, S. A., WEISS, L. M. & WITTNER, M. (1992). Chagas' disease. *Clinical Microbiology Review* **5**, 400–419.

TAYLOR, M. A. & DAVIES, H. V. (1994). Nucleotide sequence of a cDNA clone for a 60S ribosomal protein L27 gene from potato (*Solanum tuberosum* L.). *Plant Physiology* **105**, 1025–1026.

VAN VOORHIS, W. C., BARRETT, L., KOELLING, R. & FARR, A. G. (1993). FL-160 proteins of *Trypanosoma cruzi* are expressed from a multigene family and contain two distinct epitopes that mimic nervous tissues. *Journal of Experimental Medicine* **178**, 681–694.

WORLD HEALTH ORGANIZATION (1995). *Tropical Disease Research; Progress, 1975–94: Highlights, 1993–94*, pp. 125–134. Twelfth Programme Report of the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR). World Health Organization, Geneva.

WOLF, A. H., KIRSCH, M. & WIESSNER, W. (1993). Nucleotide sequence of a cDNA encoding ribosomal protein L27 from *Chlamydobotrys stellata*. *Plant Physiology* **101**, 1123–1129.