

## CHARACTERIZATION OF A *TRYPANOSOMA CRUZI* GENOMIC FRAGMENT COMPLEMENTARY TO SEVERAL SPECIES-SPECIFIC mRNAs BUT DIFFERENT FROM THE SPLICED LEADER SEQUENCE

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We have isolated a clone of *Trypanosoma cruzi* genomic DNA, lambda 3b2-5, which contains sequences that are reiterated in the genome. Northern blot analysis showed that clone 3b2-5 hybridizes to 1,200-5,000 bases different mRNA species. The number of mRNAs species hybridized to clone 3b2-5 exceeds its coding capacity showing that this clone carries sequences that are common to several mRNAs species and conserved in the poly A(+) RNA. These sequences are not homologous to the *T. cruzi* spliced leader sequence, since clone 3b2-5 does not hybridize to a synthetic 20 nucleotide complementary to the spliced leader sequence. Clone 3b2-5 does not hybridize to DNA and RNA from several genera of *Trypanosomatidae* and other *Trypanosoma* species indicating that it carries *T. cruzi* species-specific sequences.

Key words: *Trypanosoma cruzi* – genomic clones – species-specific sequences – repetitive DNA – transcriptional properties

Several families of reiterated sequences have been described in trypanosomes: the spliced leader sequence, the satellite-like repetitive sequences, the 76 bp repeat sequences found at the 5' terminus of variable surface glycoprotein genes and the telomeric repeat sequences (Boothroyd & Cross, 1982; Sloof et al., 1983; Gonzalez et al., 1984; Campbell et al., 1984; Blackburn & Challoner, 1984; Van der Ploeg et al., 1984). Recently, repetitive DNA sequences that are transcribed into a large number of poly A(+) transcripts have been observed in several different organisms including trypanosomes (Davidson & Posakony, 1982; Hasan et al., 1984; Murphy et al., 1987). The RIME (ribosomal mobile element) and TRS (trypanosome repeated sequence) sequences, transposable elements found in *Trypanosoma brucei*, are actively transcribed in the poly A(+) fraction (Hasan et al., 1984; Murphy et al., 1987). Murphy et al. (1987), established that some TRS elements may encode a protein homologous to reverse transcriptase and suggested that these sequences could be involved in the dispersion of genetic information as well

as in the shaping and reshaping of the trypanosome genome.

Here we describe the isolation and characterization of a *T. cruzi* genomic fragment that carries sequences homologous to a large number of mRNAs. These sequences are specific for *T. cruzi* and are not related to the spliced leader sequence. Our results suggest that they belong to a new family of DNA sequences characteristic of the *T. cruzi* genome.

### MATERIALS AND METHODS

*Organisms and growth conditions* – *Trypanosoma cruzi* G, Y and CL strains, *Trypanosoma mega*, *Endotrypanum schaudinni* and *Leptomonas samueli* were obtained from cultures in liver infusion tryptose (LIT) medium at exponential growth phase (Camargo, 1964). Metacyclic trypomastigotes were isolated from cultures at late stationary growth phase by chromatography on DEAE-cellulose columns. *Leishmania mexicana amazonensis*, *Crithidia deanei*, *Crithidia fasciculata*, *Crithidia luciliae termophila* and *Herpetomonas samuelpessoai*, were grown in a complex medium containing yeast extract, trypticase and sucrose (Roitman et al., 1972). *Trypanosoma theileri* and *Phytomonas davidi* were cultivated in blood agar base/LIT biphasic medium.

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**Isolation of Nucleic Acids** – High molecular weight genomic DNA was isolated from a nuclear enriched fraction of epimastigotes by the Sarkosyl/Proteinase-K/Phenol method (Maniatis et al., 1982). Total cellular RNA was isolated from epimastigotes and metacyclic trypomastigotes by the SDS/Phenol technique (Cummings et al., 1987). The RNAs were further purified by centrifugation through a cushion of 5.7M CsCl (Maniatis et al., 1982). Poly A(+) RNA was isolated by oligo-(dT) cellulose column chromatography (Pharmacia/PL, type 7). DNA and RNA from other trypanosomatids used in this study were obtained by the same procedure for *T. cruzi*.

**Construction of genomic library** – In order to construct the *T. cruzi* DNA library, nuclear DNA from epimastigotes was partially digested with EcoRI restriction endonuclease and the resulting fragments sized on sucrose gradients. The EcoRI fragments migrating between the 9.6 and 23.1 Kb lambda HindIII markers were isolated and inserted into EcoRI sites of lambda EMBL-4 (Frischauf et al., 1983). About  $5 \cdot 10^5$  independent recombinant clones per  $\mu\text{g}$  of phage DNA were obtained in host lysogenic for  $P_2$  (Frischauf et al., 1983).

**Southern and Northern hybridizations** – Southern blots were performed as described earlier (Maniatis et al., 1982). DNA samples were electrophoresed in 0.7% (W/V) agarose gels and transferred to nitrocellulose filters (Millipore). The DNA was fixed to the filter by incubation at 80 °C under vacuum for 2 h. Pre-hybridization was performed at 65 °C for 4 h in hybridization buffer [5X SSC (1 x SSC = 0.15M NaCl/0.015M Sodium Citrate), pH7.5, 5X Denhardt's solution, 0.1% SDS, 100  $\mu\text{g ml}^{-1}$  sonicated herring sperm DNA, 50  $\mu\text{g ml}^{-1}$  yeast tRNA and 10  $\mu\text{g ml}^{-1}$  poly A]. For hybridization, the incubation at 65 °C was continued for 16 h with 5-10 ml fresh hybridization solution per filter supplemented with  $2 \cdot 10^6$  cpm  $\text{ml}^{-1}$  of the appropriate probe. Post-hybridization washing conditions are described in the figure legends.

DNA dot blots were performed essentially as described in reference (Mason & Williams, 1985). The hybridization was done as described above for the Southern blots; the hybridization temperature and post-hybridization washing conditions are specified in the figure legends. DNAs from recombinant lambda EMBL-4

phages different from 3b2-5 (Fig. 4) were also blotted onto the nitrocellulose filters as hybridization controls.

Northern blots were done as previously described (Mason & Williams, 1985). RNA samples were denatured with glyoxal, electrophoresed in 1.0% (W/V) agarose gels and blotted onto nitrocellulose filters. RNA was baked onto the filter at 80 °C for 2 h. Pre-hybridization was carried out at 42 °C in formamide hybridization buffer (50% formamide, 5X SSC, 5X Denhardt's, 0.1% SDS, 100  $\mu\text{g ml}^{-1}$  sonicated herring sperm, 50  $\mu\text{g ml}^{-1}$  yeast tRNA and 10  $\mu\text{g ml}^{-1}$  poly A). The hybridization was carried out at 42 °C for 24-48 h with 5-10 ml of fresh formamide hybridization solution per filter supplemented with  $2 \cdot 10^6$  cpm  $\text{ml}^{-1}$  of the probe. Post-hybridization washing conditions are described in the figure legends.

## RESULTS

**Clone 3b2-5 carries sequences homologous to several *T. cruzi* mRNAs** – A *T. cruzi* genomic library was screened with  $^{32}\text{P}$ -labelled cDNA transcribed from poly A(+) RNAs of epimastigote and metacyclic forms. Clone 3b2-5, gave a strong positive signal with both probes.

The DNA from clone 3b2-5 was labelled *in vitro* by nick-translation and hybridized to *T. cruzi* RNAs previously fractionated on agarose/glyoxal gels and blotted onto nitrocellulose filters. It hybridized to a very large number of different mRNA species of sizes ranging from 1,200 to 5,000 bases (Fig. 1-A). The integrity of RNA preparations used in this experiment was assessed by hybridizing the filters with a *T. cruzi* genomic fragment that contains alpha and beta-tubulin genes and another genomic fragment (clone 5c), which strongly hybridizes with  $^{32}\text{P}$ -cDNA probes. The tubulin genomic fragment recognized two RNAs (Fig. 1-B), with mobilities corresponding to alpha and beta-tubulin mRNAs (Rondinelli et al., 1986). Clone 5c hybridized with a major band of 1.6 Kb (Fig. 1-C). The high molecular weight faint band in lane 1 of Fig. 1-C probably corresponds to a pre-mRNA since it is not present in the poly A(+) RNA fraction (Fig. 1-C, lane 2). When translated in an *in vitro* system, *T. cruzi* mRNAs isolated by the same technique used in this work yielded polypeptides of up to 180 kDa (Cummings et al., 1987).

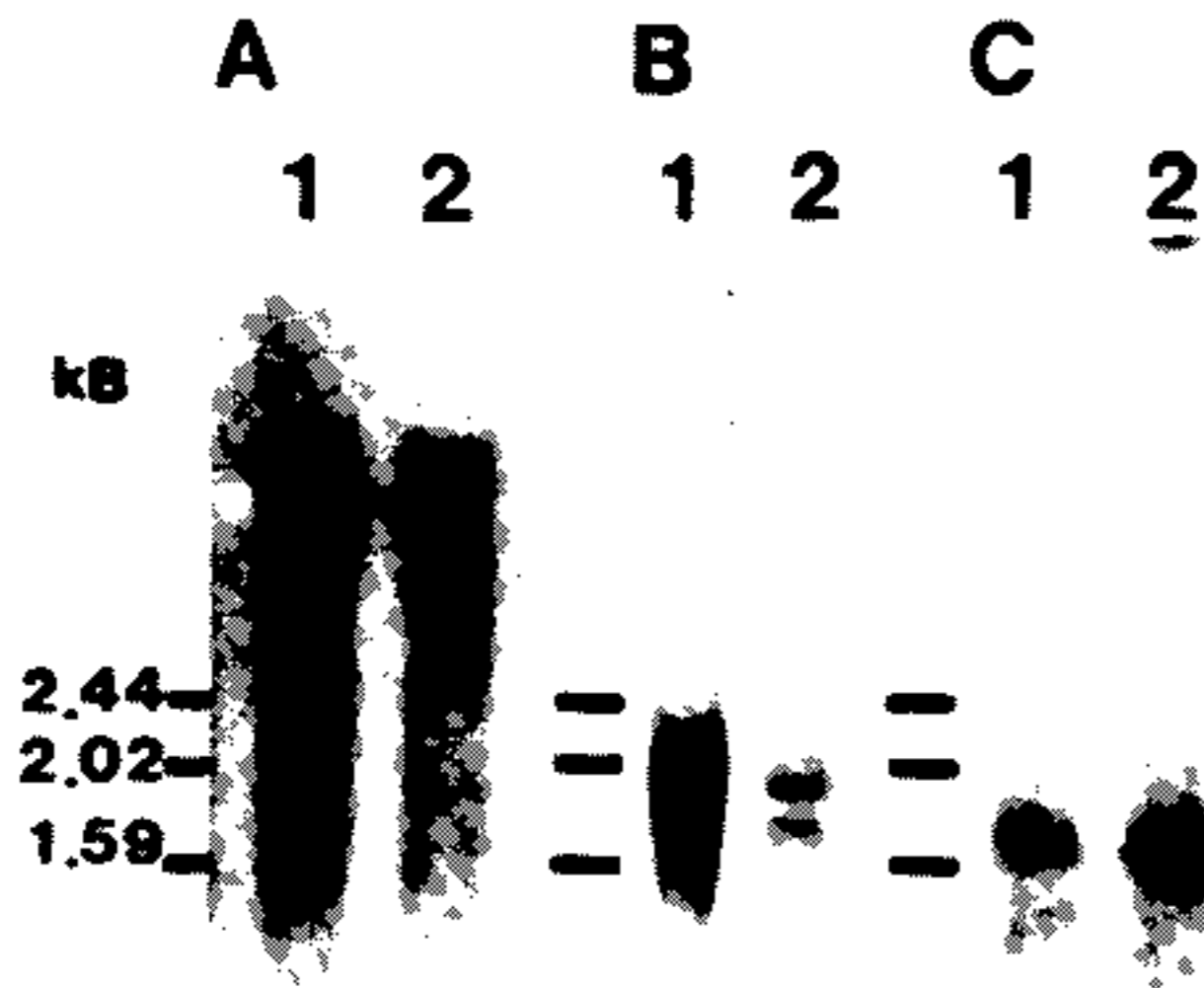


Fig. 1: *Trypanosoma cruzi* mRNA species complementary to clone 3b2-5. Total cellular RNA was extracted from epimastigote forms (CL strain) and purified by centrifugation through a cushion of CsCl. The poly A(+) fraction was obtained by chromatography on oligo-(dT) cellulose. The RNAs were size fractionated on agarose/glyoxal gels and blotted onto nitrocellulose filters. The filters were hybridized at 42 °C to nick-translated DNA from clone 3b2-5 (A); clone pYTE, a *T. cruzi* genomic fragment that carries alpha and beta tubulin genes (B) and clone 5c, a *T. cruzi* genomic fragment isolated with cDNA probes (C). Post hybridization washes were carried out in over 2.5 h at 45 °C in 0.1X SSC containing 0.1% SDS with 4 changes of buffer. Lanes: 1, total cellular RNA (8.0 µg); 2, total polyadenylated RNA (2.0 µg). Numbers on the left of the figure indicate the position of *T. cruzi* ribosomal RNAs.

**Sequences of the clone 3b2-5 complementary to *T. cruzi* mRNAs** – A partial restriction map of clone 3b2-5 is given in Fig. 2-A. The position of the restriction sites were deduced from single and multiple digestions with restriction endonucleases. Clone 3b2-5 contains an EcoRI insert 13.9 Kb long. There are two internal sites for HindIII, BamHI and Sall. In order to identify sequences of clone 3b2-5 complementary to the *T. cruzi* mRNAs, 3b2-5 DNA digested with restriction endonucleases was hybridized to labelled cDNA transcribed from epimastigote mRNAs (Figs 2-B and C). The BamHI (4.6 Kb) and BamHI/Sall (6.0 Kb) fragments hybridized heavily to the cDNA probe (Fig. 2-C, lane 3). There was no hybridization with the 2.7 Kb Sall/EcoRI fragment (Fig. 2-C, lanes 1 and 3). When larger amounts of 3b2-5 DNA were loaded onto the gel, a 0.5 Kb fragment expected after Sall digestion (see map in Fig. 2-A), was visualized after

ethidium bromide staining. This fragment hybridized to labelled *T. cruzi* DNA but not to the cDNA probe (data not shown).

**The genomic organization of 3b2-5 sequences** – To study the genomic organization of the sequences present in clone 3b2-5, the *T. cruzi* nuclear DNA was digested with several restriction endonucleases and hybridized to nick-translated DNA from clone 3b2-5. Clone 3b2-5 hybridized to a large number of genomic fragments and therefore contains sequences that are reiterated in the *T. cruzi* genome (Fig. 3).

**The 3b2-5 sequences are not homologous to the *T. cruzi* spliced leader sequence** – A common short leader sequence of 35 nucleotides is found at the 5' end terminus of many, if not all, mRNAs in trypanosomes (Boothroyd & Cross, 1982; DeLange et al., 1984; Milhausen et al., 1984; Nelson et al., 1984; Van der Ploeg et al., 1982). To determine whether the sequences contained in clone 3b2-5 were identical to the spliced leader sequence found on mRNAs, we hybridized the clone 3b2-5 with a synthetic 20 nucleotide complementary to the *T. cruzi* spliced leader sequence (DeLange et al., 1984; Milhausen et al., 1984) (Fig. 4). The synthetic oligomer was <sup>32</sup>P-5' end-labelled and hybridized to clone 3b2-5 under mild stringent conditions as described earlier (Milhausen et al., 1984). The <sup>32</sup>P-oligomer hybridized to *T. cruzi* DNA but not to clone 3b2-5 (Fig. 4). Since in this experiment we used equivalent amounts of *T. cruzi* and phage DNAs (1.25 µg each), we can conclude that clone 3b2-5 does not contain sequences homologous to the spliced leader sequence.

Genomic sequences homologous to the *T. cruzi* spliced leader sequence are located in 0.6 Kb units arranged in tandem repeats (DeLange et al., 1984; Nelson et al., 1984). A labelled synthetic nucleotide complementary to the spliced leader sequence hybridizes to a 0.6 Kb fragment obtained by complete digestion of *T. cruzi* DNA with Pst I or Mbo I (DeLange et al., 1984; Nelson et al., 1984). The *T. cruzi* DNA was digested with either Mbo I or Pst I and hybridized to labelled DNA from clone 3b2-5 (Fig. 3, lanes 3, 4, 7 and 8). There was no hybridization below the 1.5 Kb region of the gel suggesting that the clone 3b2-5 does not carry sequences coding for the *T. cruzi* spliced leader sequence.

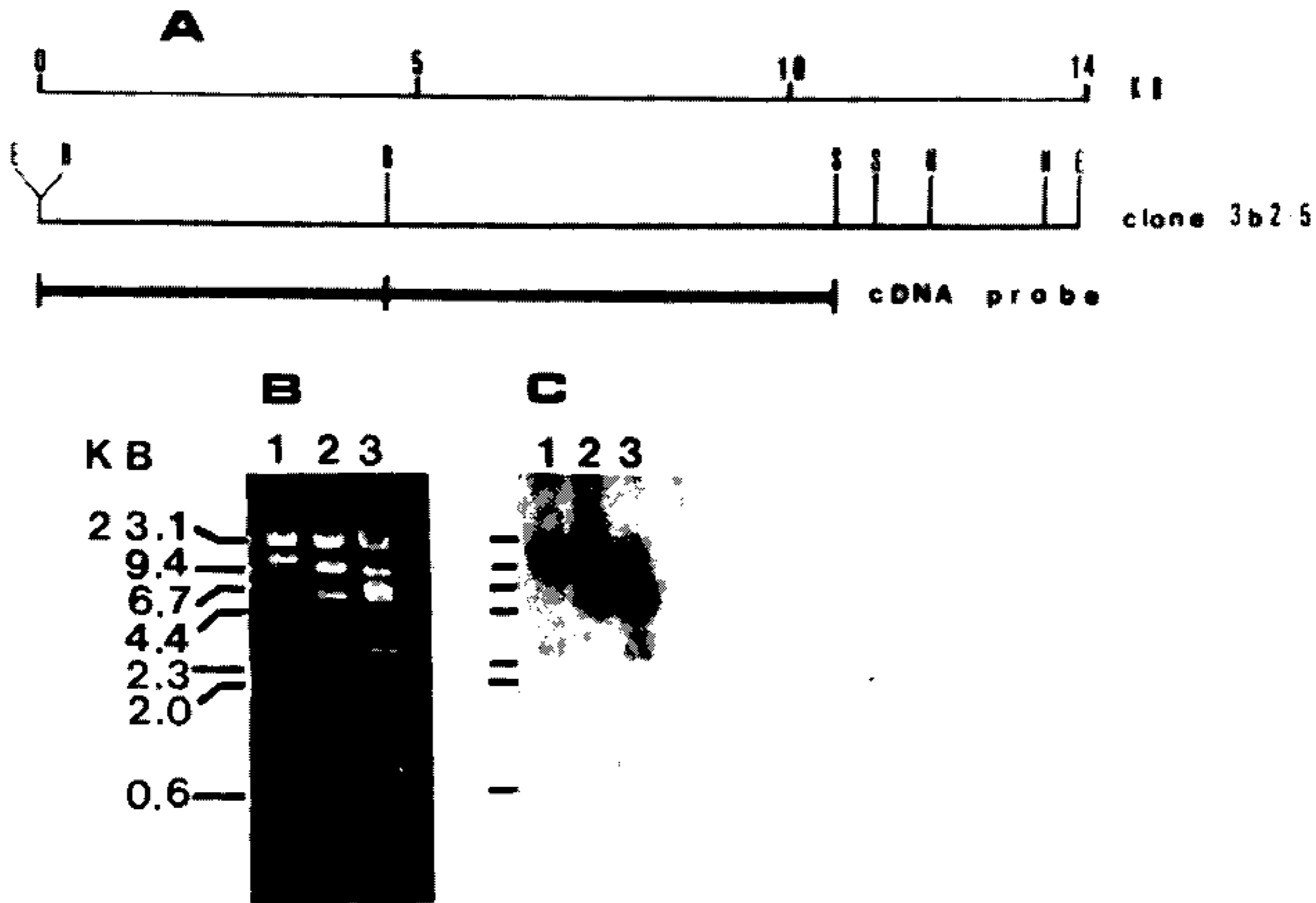


Fig. 2: sequences of the clone 3b2-5 complementary to the *T. cruzi* mRNAs. (Panel A) Restriction map of clone 3b2-5. The position of the restriction sites were deduced from single and multiple digestions with restriction endonucleases. The heavy line indicates the region homologous to the cDNA probe. E, EcoRI; B, BamHI; S, Sall; H, HindIII. (Panels B, C) The 3b2-5 DNA was digested with several restriction endonucleases, size fractionated on agarose gels and stained with ethidium bromide (B). The digested fragments were blotted onto nitrocellulose filters and hybridized at 65 °C to labelled cDNA transcribed from epimastigote poly A(+) mRNAs (C). Post-hybridization washes were carried out at 65 °C two times for 30 min in 2X SSC, twice for 30 min in 1X SSC and for 60 min in 0.1X SSC. Lanes: 1, EcoRI and Sall; 2, EcoRI and BamHI; 3, EcoRI, Sall and BamHI. Numbers on the left of the figure indicate the position of lambda DNA fragments after digestion with HindIII.

The 3b2-5 sequences are species-specific for *T. cruzi* — We have also analyzed several genera of Trypanosomatidae and *Trypanosoma* species looking for sequences homologous to clone 3b2-5 by dot-blot hybridization, Southern and Northern blot analyses. DNAs from several trypanosomatids (*Phytomonas*, *Leishmania*, *Crithidia*, *Endotrypanum*, *Leptomonas Herpetomonas* and *Trypanosoma theileri*) were hybridized to nick-translated DNA from clone 3b2-5 under mild stringent conditions (Fig. 5). The probe hybridized exclusively with the *T. cruzi* DNAs suggesting that clone 3b2-5 carries species-specific sequences. Interestingly, clone 3b2-5 did not hybridize to DNAs of other *Trypanosoma* species such as *Trypanosoma theileri* (Fig. 5), *Trypanosoma mega* and *Trypanosoma equiperdum* (data not shown).

The results described above were confirmed hybridizing the labelled 3b2-5 DNA with RNAs from different trypanosomatids (*Leishmania*, *Leptomonas*, *Herpetomonas*, *Crithidia* and *Trypanosoma mega*). The probe hybridized

exclusively to *T. cruzi* RNAs (Fig. 6). The hybridization patterns of clone 3b2-5 to RNAs from different *T. cruzi* strains are similar to each other (Fig. 6, lanes 6 and 8).

#### DISCUSSION

Recombinant clones strongly reactive to epimastigote and trypomastigote cDNAs were selected in a *T. cruzi* genomic library. We have characterized one of such clones designated 3b2-5, which carries sequences common to many mRNAs species, conserved in the poly A(+) fraction and not homologous to the *T. cruzi* spliced leader sequence.

Trypanosome mRNAs have a 35 nucleotide 5' leader sequence encoded by a tandemly repeated mini-exon (DeLange et al., 1984; Milhausen et al., 1984; Nelson et al., 1984). DNA from clone 3b2-5 did not hybridize to a synthetic 20 nucleotide probe complementary to the *T. cruzi* spliced leader sequence (Fig. 4). In addition, it is known that the 35 nucleotide

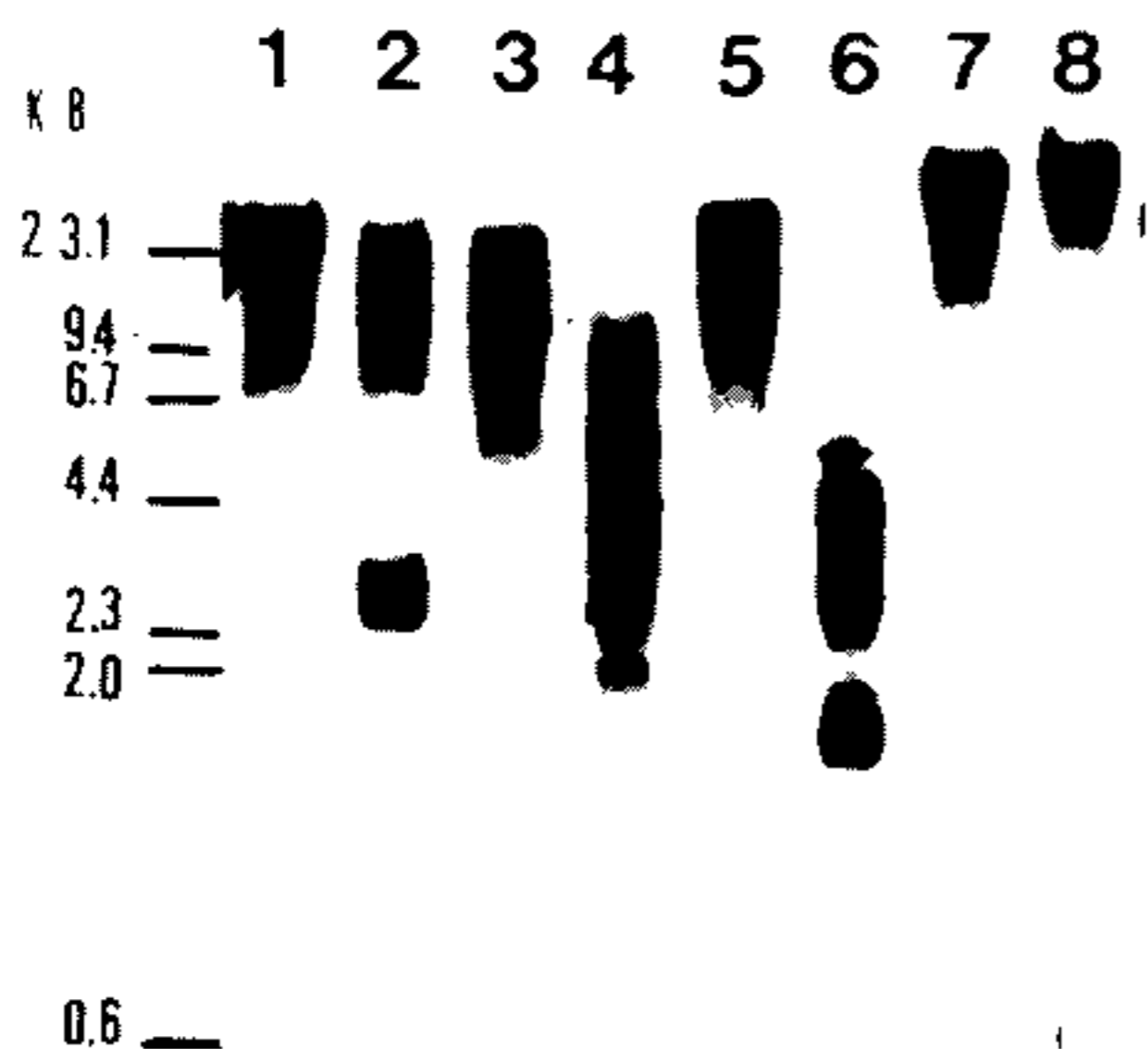


Fig. 3: genomic organization of the sequences present in clone 3b2-5. The nuclear DNA of epimastigote forms (5.0  $\mu$ g) was digested with restriction endonucleases and size fractionated on agarose gels. The restricted fragments were blotted onto nitrocellulose filters and hybridized at 65 °C to nick-translated DNA from clone 3b2-5. The post-hybridization washing conditions are described in legend of the figure 2. Lanes: 1, EcoRI, partial digestion; 2, EcoRI, total digestion; 3, Mbo I, partial digestion; 4, Mbo I, total digestion; 5, Alu I, partial digestion; 6, Alu I, total digestion; 7, Pst I, total digestion; 8, Pst I, partial digestion. Numbers on the left of the figure indicate the position of lambda DNA fragments after digestion with HindIII.

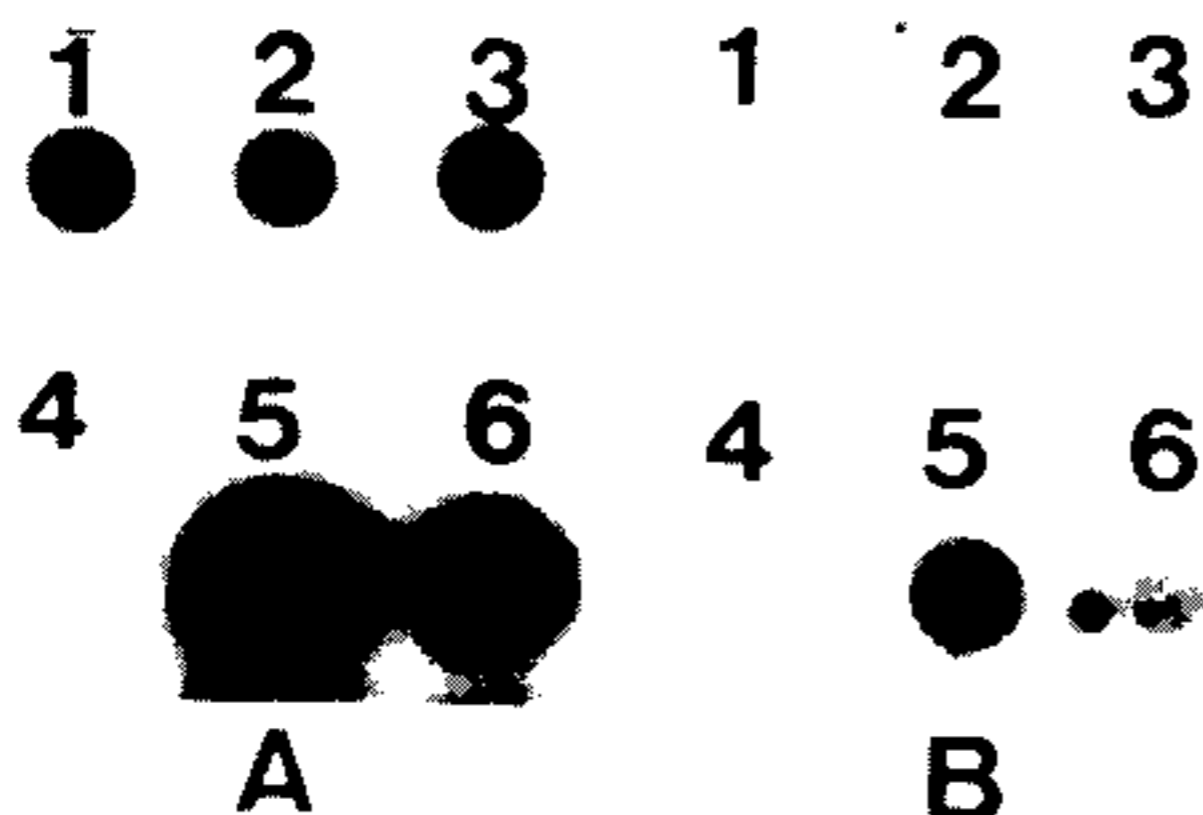


Fig. 4: labelled synthetic nucleotide oligomer complementary to the *T. cruzi* spliced leader sequence does not hybridize to clone 3b2-5. DNAs from recombinant phages (clones 3b2-5 and 9a2), vector and *T. cruzi* were denatured by heat and bound to nitrocellulose filters. The filters were hybridized to the labelled *T. cruzi* nuclear DNA (A), as described in legend of the figure 2, and to labelled synthetic 20 nucleotide complementary to the *T. cruzi* spliced leader sequence (B). The hybridization with labelled synthetic nucleotide oligomer was carried out at 35 °C for 24 h in 5X SSC. After hybridization the blot was washed twice at 25 °C and twice at 35 °C in the hybridization solution. Dots: 1 and 2, clone 3b2-5 (1.25  $\mu$ g); 3, clone 9a2 (1.25  $\mu$ g); 4, non recombinant phage EMBL-4 (1.25  $\mu$ g); 5, *T. cruzi* DNA (1.25  $\mu$ g); 6, *T. cruzi* DNA (0.12  $\mu$ g).

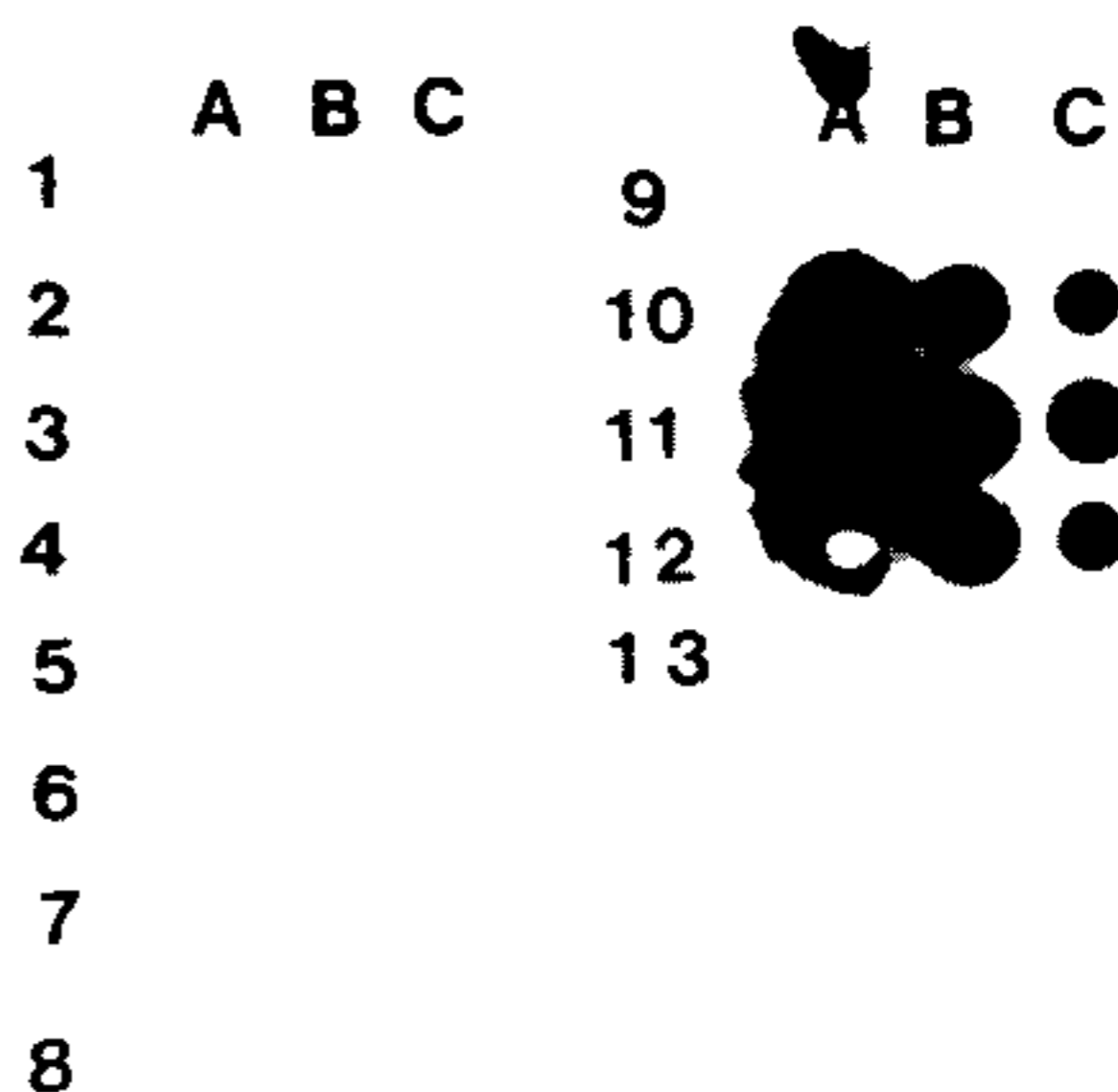


Fig. 5: demonstration of species-specificity of 3b2-5 sequences in dot-blot hybridization experiments. DNAs were extracted from trypanosomatid culture forms by sarkosyl/proteinase-K/phenol procedure, denatured by heat and bound to nitrocellulose filters. The filters were hybridized at 50 °C to nick-translated 3b2-5 DNA. Post-hybridization washes were carried out at 55 °C once for 30 min in 5X SSC containing 0.1% SDS and three times for 30 min in 2X SSC containing 0.1% SDS. Columns A to C represent a 10-fold dilution series (10  $\mu$ g, 1  $\mu$ g and 0.1  $\mu$ g, respectively) of trypanosomatid DNAs. Rows: 1, *Phytomonas davidi*; 2, *Leishmania mexicana amazonensis*; 3, *Crithidia fasciculata*; 4, *Endotrypanum schaudinni*; 5, *Crithidia luciliae termophila*; 6, *Leptomonas samueli*; 7, *Herpetomonas samuelpessoai*; 8, *Crithidia deanei*; 9, *Trypanosoma theileri*; 10, *Trypanosoma cruzi*, Y strain; 11, *Trypanosoma cruzi*, G strain; 12, *Trypanosoma cruzi*, CL strain; 13, salmon sperm DNA.

leader sequence and the cloned *T. cruzi* spliced leader mini-exon hybridize to a 0.6 Kb fragment obtained by digestion of *T. cruzi* genomic DNA with either PstI or MboI (DeLange et al., 1984; Nelson et al., 1984). Fig. 3 shows clearly that the clone 3b2-5 did not recognize the 0.6 Kb fragment obtained by digestion of *T. cruzi* DNA with these enzymes. Based on these arguments, we can conclude that the 3b2-5 does not carry sequences homologous to the *T. cruzi* spliced leader sequence.

Clone 3b2-5 carries sequences that are reiterated in *T. cruzi* genome. When Southern blots carrying *T. cruzi* DNA digested with several endonucleases were hybridized to the labelled 3b2-5 DNA, multiple sequences were recognized by the probe (Fig. 3). The probe hybridized to several *T. cruzi* fragments obtained by digestion with EcoRI endonuclease. An EcoRI fragment of around 14 Kb corresponds in size to the genomic fragment cloned in phage 3b2-5. As this enzyme does not

cut within the clone 3b2-5 (Fig. 2-A), we can suggest that most of the sequences complementary to clone 3b2-5 are interspersed in the genome rather than in tandem repeats.

The transcripts homologous to clone 3b2-5 range in size between 1.2 and 5.0 Kb (Figs 1 and 6). No discrete size class of transcripts was observed suggesting that mRNA molecules recognized by clone 3b2-5 are not partially processed precursors from the same RNA, as described earlier (Gonzalez et al., 1985). The transcriptional properties of clone 3b2-5 resemble those described for *Trypanosoma brucei* retroposons (Hasan et al., 1984; Murphy et al., 1987). The RIME and TRS elements are composed of reiterated interspersed sequences complementary to a large number of *T. brucei* mRNAs and conserved in the polyadenylated fraction. These retroposons do not contain the mini-exons coding for the spliced leader sequence.

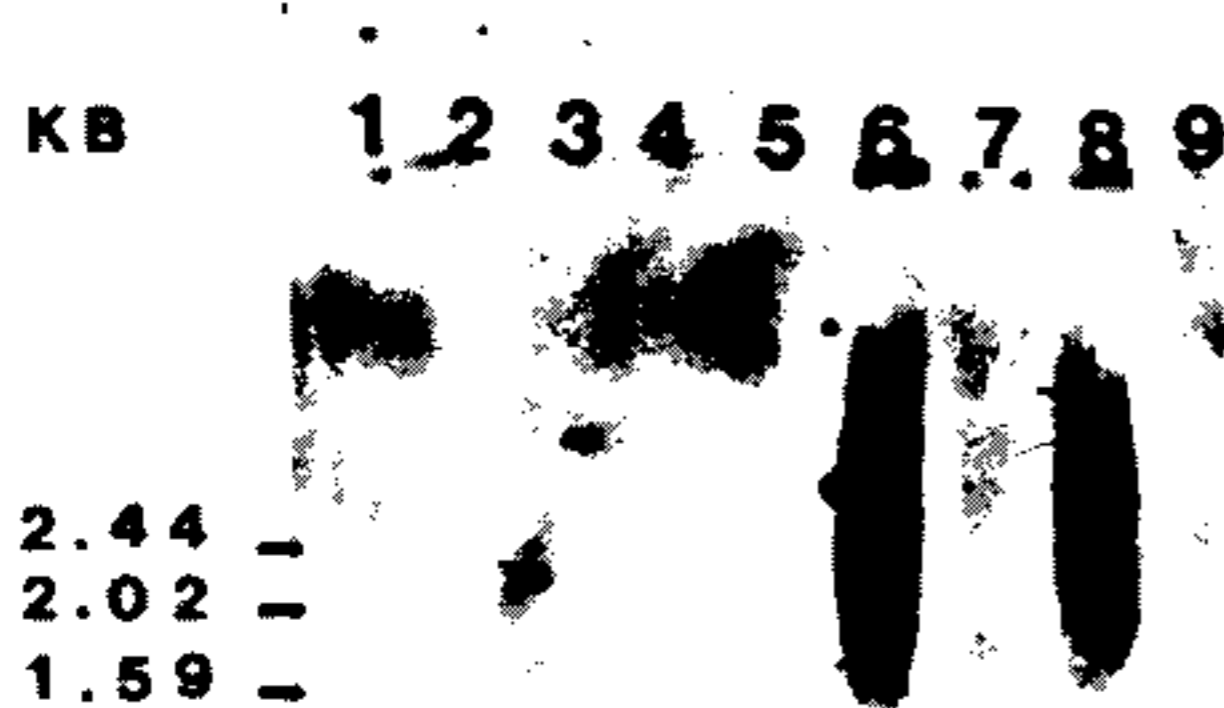


Fig. 6: distribution of 3b2-5 sequences in different *T. cruzi* strains and related trypanosomatids. Total cellular RNAs were extracted from culture forms and purified by centrifugation through a cushion of CsCl. 8  $\mu$ g of each RNA (except for *T. cruzi*, Y strain, where just 0.5  $\mu$ g was used) were size fractionated on agarose/glyoxal gels and blotted onto nitrocellulose filters. The filters were hybridized at 42 °C to nick-translated DNA from clone 3b2-5. Post-hybridization washes were carried out for 2.5 h at 42 °C in 2X SSC containing 0.1% SDS with 4 changes of buffer. Numbers on the left of the figure indicate the position of *T. cruzi* ribosomal RNAs. Lanes: 1, *Leishmania mexicana amazonensis*; 2, *Leptomonas samueli*; 3, *Trypanosoma mega*; 4, *Crithidia deanei*; 5, *Herpetomonas samuelpessoai*; 6, *Trypanosoma cruzi*, G strain; 7, *Trypanosoma cruzi*, Y strain; 8, *Trypanosoma cruzi*, CL strain; 9, total mouse liver RNA.

Another aspect studied in this work was the distribution of 3b2-5 sequences in different trypanosomatids. The sequences present in clone 3b2-5, abundant in *T. cruzi*, are absent in all trypanosomatids and related trypanosome species examined (Figs 5 and 6). One of the possible explanations for these results could be a recent evolutionary origin and amplification of these sequences in *T. cruzi*.

The abundance of 3b2-5 sequences in transcripts from different *T. cruzi* developmental forms might suggest that these sequences have a function in the trypanosome genome. Analysis of cDNA clones of 3b2-5 should help to address some questions regarding this recombinant clone and their transcripts. For instance: a) Are the transcripts homologous to clone 3b2-5 capped by the spliced leader sequence?, b) What is the organization of the 3b2-5 sequences in the cDNA clones?

We are currently carrying out experiments to subclone the 4.6 Kb BamHI fragment of clone 3b2-5 in order to determine its nucleotide sequence. This fragment will be used to isolate cDNA clones of 3b2-5 in *T. cruzi* cDNA libraries.

#### RESUMO

**Caracterização de um fragmento genômico de *Trypanosoma cruzi* complementar a diferentes espécies específicas de ARNs mensageiros mas diferente da seqüência líder** – Foi isolado um clone recombinante ( $\lambda$  3b2-5) de ADN genômico de *Trypanosoma cruzi*, o qual contém seqüências que estão reiteradas no genoma. Análise através da técnica de "Northern blot" mostrou que o clone 3b2-5 hibridiza com diferentes espécies de ARNs mensageiros de 1.200 a 5.000 bases. O número de espécies de ARNs mensageiros que hibridiza com o clone 3b2-5 excede sua capacidade codante, sugerindo que este clone apresenta seqüências que são comuns a muitas espécies de ARNs mensageiros e conservadas no ARN poliadenilado. Estas seqüências não são homólogas à seqüência líder presente nos ARNs de *T. cruzi*, pois, o clone 3b2-5 não hibridiza com um nucleotídeo sintético complementar à seqüência líder. O clone 3b2-5 não hibridiza com o ADN e ARN de diferentes gêneros de tripanosomatídeos e outras espécies de tripanosomas indicando que ele apresenta seqüências específicas para o *T. cruzi*.

Palavras-chave: *Trypanosoma cruzi* – clones genômicos – seqüências espécie-específicas – ADN repetitivo – propriedades transcricionais

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