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CLONACION MOLECULAR Y CARACTERIZACION PARCIAL DEL DNA RIBOSOMAL DE *TRYPANOSOMA CRUZI*

Tesis que para obtener el grado de

DOCTOR EN INVESTIGACION BIOMEDICA BASICA

Presenta:

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ANTECEDENTES GENERALES

Trypanosoma cruzi es un protozoario flagelado capaz de parasitar al hombre y causar en el la enfermedad de Chagas. Esta última, también conocida como tripanosomiasis americana, es un padecimiento crónico y debilitante que afecta en Centro y Suramérica a unos 20 millones de personas |1| . En la República Mexicana se ha encontrado al insecto vector (género *Triatoma*) infectado, en casi todos los estados del territorio nacional |2|; lo que hace suponer que la zona endémica sea muy amplia. En relación al número de personas parasitadas, cabe mencionar que en algunas localidades de Chiapas se encontró un 20% de individuos escogidos aleatoriamente con serología positiva a *T. cruzi* |3| .

El padecimiento se transmite por la presencia del parásito en la sangre de un individuo infectado. En medios urbanos, es frecuente que la infección se lleve a cabo por transfusiones con sangre contaminada. En condiciones rurales, por otro lado, participan insectos hematófagos de los géneros *Triatoma*, *Rhodnius* ó *Pastrongilus*.

Formas del parásito y ciclo de vida

Al igual que otros protozoarios parásitos, *T. cruzi* cursa tanto en el hombre como en el insecto vector por un ciclo de vida

complejo que incluye diferenciación celular con transformación a estadios identificables morfologicamente. Dichas formas, incluyen células redondeadas (amastigotes), células ovoides (epimastigotes)y formas alargadas llamadas tripomastigotes |4|. Inicialmente se consideró que los amastigotes eran formas aflageladas, sin embargo, estudios posteriores de ultraestructura indicaron la existencia de un flagelo incipiente. Los epimastigotes presentan un flagelo visible en microscopía óptica, y al igual que en las formas amastigotas, la emergencia de éste se localiza posterior al núcleo. Los tripomastigotes, por otro lado, fueron descritos inicialmente como formas del parásito con una membrana ondulante. Sin embargo, estudios de microscopía electrónica, mostraron que la emergencia del flagelo a diferencia de las formas anteriores se localiza hacia la punta de la célula por delante del núcleo, y al regresar por fuera del cuerpo, adosado a la membrana citoplasmática da la imágen de una membrana ondulante.

Existe una íntima correlación entre la posición de emergencia del flagelo relativa al núcleo, y la ubicación de una estructura refringente llamada cinetoplasto. Esta estructura esta constituída por una red concatenada de DNA, y su ubicación dentro de la célula se limita al espacio interior de la mitocondria única del parásito. El cinetoplasto, además del flagelo, es característico del orden cinetoplástida, y su nombre deriva inicialmente de la idea que podría ser un plástido involucrado en la regulación del movimiento flagear.

Después de haber descrito las características más generales de morfología que identifican a los amastigotes, epimastigotes y a los tripomastigotes; quisiera referirme a algunos aspectos generales de su ciclo de vida que reflejan su fisiología diferente.

Los tripomastigotes en el hospedero mamífero, representan la forma sanguínea y extracelular del parásito, y constituyen el único estadio de diferenciación en que T. cruzi es capaz de invadir células. Estas formas tripomastigotas no se dividen, y en este sentido son terminales, a menos de que infecten alguna célula del hospedero. En esta dirección, después de alcanzar el espacio intracelular, los tripomastigotes se transforman en las formas amastigotas y se dividen por fisión binaria cientos de veces. En algún momento y aún dentro de la célula, los amastigotes se diferencían a nuevos tripomastigotes, lisan a la célula (posiblemente por ruptura mecánica), y se liberan al torrente circulatorio donde pueden reiniciar los ciclos de invasión, desdiferenciación con replicación y lísis celular. Aunque se han descrito eventos de autoinmunidad |5|, es generalmente aceptado que la lísis tisular es la responsable de la fisiopatología del padecimiento. Así se tiene que al destruír tejido nervioso que inerva los órganos huecos del aparato digestivo, se pierde la tonicidad de los mismos dando lugar al llamado síndrome de mega esófago, mega estómago y mega colon. Por otro lado, si el órgano blanco es el corazón, la patología que se presenta es la contracción arritmica del mismo.

En el vector, el ciclo de vida se inicia cuando el insecto ingiere sangre de un individuo parasitado. Las formas sanguíneas tripomastigotas, ahora en el vector, adquieren a lo largo de la luz intestinal del insecto y aparentemente sin infectar células, formas replicativas de epimastigotes. En forma análoga a lo descrito con anterioridad, los epimastigotes se diferencían a tripomastigotes en la porción distal del intestino del insecto infectado. Estos tripomastigotes últimos, se les denomina metacíclicos por haber completado un ciclo de diferenciación distinto al del hospedero mamífero.

El vector transmite el padecimiento cuando a consecuencia de una nueva ingestión sanguínea el insecto infectado defeca _in situ_ tripomastigotes metacíclicos. Si por alguna razón estas formas alcanzan conjuntivas, mucosas o el torrente sanguíneo, los ciclos de invasión y lísis celular se llevarán a cabo con el consecuente establecimiento de la parasitosis.

El hecho de que _T_. _cruzi_ se replique intracelularmente en el hospedero mamífero, constituye quizá el mecanismo mas importante de evasión de la respuesta inmunológica, pues si bien el huésped es capaz de montar una respuesta inmune tanto celular como humoral |6| que elimina del torrente circulatorio a la mayoría de tripomastigotes durante la fase crónica de la enfermedad, el aparato inmunológico no alcanza los seudoquistes intracelulares de amastigotes replicantes.

Por otro lado, es interesante que a pesar de que el parásito es morfológicamente similar, las características del padecimiento, en lo que se refiere a la intensidad y tipo de tejido afectado, varían dependiendo de la localización geográfica donde éste es aislado |4,7|. Así se tiene que mientras en Brazil las parasitosis se presentan con problemas digestivos y cardiacos, en Venezuela predominan los cuadros clínicos con alteraciones cardiacas |7|. Estas manifestaciones diferentes de la enfermedad de Chagas pueden deberse a diferencias en los enfermos y/o a diferencias en el parásito. Considerando la posibilidad, cabe mencionar que existen cepas de ratones que son diferencialmente sensibles al tripanosomatídeo Leishmania donovani y que esta sensibilidad esta determinada geneticamente |8|. En lo referente a la posibilidad de diferencias entre los parásitos, mencionaré que a través de analizar el patrón electroforético de algunas isoenzimas de T. cruzi, fue posible la clasificación de éste en grupos o zimodemas. Resulta interesante que dichos zimodemas se encuentren diferencialmente distribupidos en Venezuela y en Brazil |7|.

T. cruzi ha sido estudiado con diferentes enfoques: Existen revisiones sobre su fisiología |9|, aspectos de estructura celular |10|. y sobre biología molecular |11|. De igual manera existe literatura abundante sobre la respuesta inmune del hospedero |6|.

Organización del genoma

En el contexto de la organización genómica, el DNA mas estudiado es el DNA del cinetoplasto; que dependiendo de la especie puede representar entre el 10 y el 20% del DNA total celular |11|. En T. cruzi el trabajo inicial mas importante sobre este DNA, fue realizado por el grupo de G. Riou |12|, quién demostró su arreglo supramolecular como una red concatenada de 30,000 a 50,000 moléculas circulares, compuestas individualmente de aproximadamente 1400 pares de bases (minicírculos). Los patrones generados por digestiones enzimáticas con endonucleasas de restricción, indican que dichos minicírculos de DNA, están representados por secuencias heterogéneas. Sin embargo, se ha descrito repetitividad intrínseca de moleculas individuales.

La posible transcripción del DNA de los minicírculos ha sido motivo de controversias, y tradicionalmente se ha pensado que dicho DNA no se transcribe. El RNA mitocondrial, como RNA ribosomal y mRNA de enzimas respiratorias, proviene en el cinetoplasto de moléculas de DNA también circulares, pero de mayor longitud (20- 30kb) |13, 14|. Estas corresponden a solo un 5% del DNA presente en el cinetoplasto |11|. Sin embargo, regresando al problema que representa la transcripción del DNA de los minicírculos, se ha descrito recientemente la existencia de moléculas pequeñas de RNA mitocondrial (75 y 200b) que hibridan con DNA de los minicírculos |15|. El papel biológico de estos transcritos es aún

desconocido, pero se postula que puedan funcionar como "primeros" en la replicación del DNA de los minicírculos. En forma alternativa y por datos de secuencia, los autores postulan que dichos transcritos codificarían, en el caso de ser traducidos, para proteínas básicas capaces de asociarse al DNA con una función estructural.

A diferencia de los tripanosomas africanos, donde se han estudiado extensamente la organización y la expresión de genes nucleares que codifican tanto para los antígenos variables de superficie |16|, como para proteínas estructurales (tubulina) |17| o regulatorias (calmodulina) |18|, el estudio molecular de genes nucleares en T. cruzi, es aún incipiente. Por datos de velocidad de renaturalización, C. Castro y col han estimado que el genoma nuclear de T. cruzi contiene alrededor de 11,000 genes|19|. Dadas las características biológicas ya mencionadas, el estudio molecular de genes nucleares de T. cruzi merece una atención particular.

Elección del sistema génico de estudio

En la elección de un sistema génico nuclear accesible e interesante, consideramos que el RNA ribosomal (RNAr) citoplásmico ofrecía las siguientes características:

1. Tanto la abundancia celular del RNAr, como la repetitividad génica encontrada en diversas especies celulares |20|, lo convierten en un sistema de fácil acceso.

2. Debido, por un lado, a que la transcripción del RNAr se encuentra asociada con crecimiento celular |21, 22|, y por otro a que el parásito cursa durante su ciclo de vida por estadios replicativos y no replicativos; pensamos que muy probablemente la síntesis de RNAr se encuentra regulada diferencialmente en dichos estadios. De ser así, este sistema génico ofrece la posibilidad de estudiar secuencias nucleotídicas involucradas en la regulación de su expresión.

3. La función del RNAr es muy antigua en la evolución y ha sido mantenida en todos los tipos celulares. Por estos motivos los estudios comparativos de secuencias nucleotídicas de RNAr en distintos organismos, han resultado tan informativos en la construcción de relaciones taxonómicas y filogenéticas |23|.

ANTECEDENTES PARTICULARES

Para presentar las consideraciones particulares que antecedieron al presente trabajo de Tesis, me permito incluir en este capítulo las comunicaciones: "Small-size ribosomal RNA species in *Trypanosoma cruzi*", y "An endonuclease restriction analysis of the ribosomal RNA genes of *Trypanosoma cruzi*", producto de mi trabajo de Maestría.

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SMALL-SIZE RIBOSOMAL RNA SPECIES IN *TRYPANOSOMA CRUZI*

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Trypanosoma cruzi ribosomal RNA was analyzed by electrophoresis. On agarose gels, where both large- and small-size species are grossly fractionable, it revealed two bands in the small-size region. These were similar in size to the mammalian 5.8 S and 5 S species. Increased resolution, however, showed these two bands to be composite. The pseudo 5.8 S band contained three, and the pseudo 5 S two, discretely sized molecules. The ribosomal binding of four of these five novel species is apparently dependent on large ribosomal subunit proteins. One species is hydrogen bonded to the β species of 24 S ribosomal RNA. The five species were estimated to be 261, 217, 197, 141 and 110 nucleotides long.

Key words: *Trypanosoma cruzi*, rRNA; Pseudo 5.8 S and 5 S RNA; Small-size rRNA species

INTRODUCTION

Mature cytoplasmic ribosomal RNA (rRNA) of most higher eukaryotes is composed of two large- and two small-size species: the 23–28 S and 18 S, and the 5.8 S and 5 S molecules, respectively. Some organisms deviate from this pattern. We have reported [1] that *Trypanosoma cruzi* contains, not two, but three large-size species: two (species α and β of approximately 1700 and 2000 nucleotides, respectively) released upon denaturation of the 24 S rRNA (about 4000 nucleotides), and one species in the 18 S rRNA (about 2500 nucleotides). The data indicated also, that the α and β species most probably are due to late processing of the original rRNA transcript, rather than to nonspecific ribonuclease (RNAase) activity on the 24 S rRNA. These large-size species have also been reported in other trypanosomatids such as *Crithidia luciliae* [2], *Leishmania donovani* [3], *C. oncopelti, C. fasciculata* and *Trypanosoma brucei* [4,5]; as well as in the protozoa *Acanthamoeba castellani* [6] and *Tetrahymena pyriformis* [7,8], in several protostomia [9], and in *Drosophila melanogaster* [10].

Information concerning small-size rRNA species is now emerging. In our previous work [1], we estimated that the α and β species together contained some 300 nucleo-

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tides fewer than did 24 S rRNA. This difference was partially accounted for by the probable release, upon denaturation, of a naturally occurring small-size rRNA such as the known 5.8 S species which contains some 160 nucleotides. We have extended the work on rRNA and here we report that, in contrast to higher eukaryotes, *T. cruzi* has five small-size rRNA species. These results are similar to those recently reported for *T. brucei* [11] and *C. fasciculata* [12].

MATERIALS AND METHODS

Cell culture. T. cruzi isolated in La Cruz, Jalisco, Mexico (Dr. J. Tay, School of Medicine, UNAM) was grown in liquid medium containing liver infusion-tryptose supplemented with 10% heat-inactivated fetal calf serum, as described elsewhere [1].

Preparation of ribosomes and rRNA. Cells were collected in mid-log phase of growth (ca. 99% epimastigote forms) by centrifugation at 8000 × *g* for 15 min at 4°C and were then washed twice with 30 mM Tris-HCl (pH 7.0), 100 mM KCl, 5 mM $MgCl_2$, 3 mM $CaCl_2$ containing an RNAase-inhibiting mixture of diethylpyrocarbonate (10 μg ml^{-1}), heparin (4 mg ml^{-1}). This mixture was used in all solutions thereafter and for rinsing all glassware. Also, solutions and glassware were previously autoclaved, and gloves were worn during this and the following procedures. 1 g of wet and packed cells was resuspended in 10 ml of the washing solution and lysed by the addition of Nonidet P-40 to a final concentration of 0.3%. Under these conditions most of the nuclei remained intact (as determined by absence of viscosity in the lysate and by phase microscopy). The lysate was sequentially centrifuged at 10 000, 20 000 and 100 000 × *g* for 10, 10 and 120 min, respectively. The last pellet was used as a source of ribosomes. To extract rRNA, this pellet (or ribosomes fractionated by sucrose-gradient centrifugation, see below) was resuspended in 20 mM Tris-HCl (pH 7.0), 2.0 M LiCl, 4.0 M urea and allowed to stand at 4°C for 24 h. This suspension was centrifuged at 20 000 × *g* for 30 min, and the pellet was rinsed with, and resuspended in water. The rRNA preparations showed absorption ratios $A_{260/280}$ and $A_{260/230}$ equal to or higher than 2.0 and 2.4, respectively. The material was sensitive to 1 min-boiled pancreatic RNAase and to 0.1 M NaOH at 37°C. Paper electrophoresis of these digests showed uridine but not thymidine (unpublished experiments done in collaboration with Ms. R. Sánchez). The yield of rRNA has been around 1 mg g^{-1} of wet cells. Other rRNA isolation procedures [1] were also used and gave similar results in respect to the small size rRNA species. Mouse (Swiss albino mice) liver rRNA, used as a size marker, was obtained by the usual phenol extraction and ethanol precipitation method. *T. cruzi* rRNA was either directly electrophoresed or firstly resolved on sucrose gradients.

Sucrose gradients. Samples with ribosomal material or extracted rRNA were fractionated on nondenaturing isokinetic sucrose gradients in the absence [1], or in the presence of high K^+ concentrations to obtain ribosomal subunits [12].

Gel electrophoresis. The rRNA samples were analyzed on two types of slab gels: (a) nondenaturing gels, and (b) denaturing gels. (a) 1.5% agarose gels (4 mm thickness; 30 cm length) in 10.8 g Tris (pH 8.3), 5.49 g boric acid, 0.93 g EDTA per l, were run at 6 V cm^{-1} for 2 h at 4°C. (b) 3.5% acrylamide, 0.15% bisacrylamide gels (1.5 mm thickness; 12 cm length) in the same solution as above, but with 7 M urea, were prerun at 2 V cm^{-1} overnight; electrophoresis was at 4 V cm^{-1} for 8 h at ambient temperature. The samples of rRNA were firstly dissolved in the electrophoresis solution, containing 20% glycerol and bromophenol blue as front marker. Slab gels were stained with 3.0 μg ml^{-1} of ethidium bromide and photographed under short UV light. In some experiments, the rRNA bands were cut out and electroeluted in the same electrophoresis solution overnight at 4°C.

RESULTS

When *T. cruzi* total rRNA, obtained directly from the ribosomal pellet or from monosomes purified in nondenaturing sucrose gradients (see Methods), was analyzed under partially nondenaturing conditions (for this material) on long agarose gels, where both large- and small-size species can be fractionated (Fig. 1a), it resolved into six bands in total: four large-size bands (undenatured 24 S, 18 S, and β and α derived from denatured 24 S) already characterized [1], and two small-size bands. Fig. 1A also shows some bands with lower mobility than that of 24 S rRNA. When this material was electroeluted, denatured and reelectrophoresed on denaturing polyacrylamide gels (as in Fig. 1c and d; see below), it was resolved into the three large-size 18 S, β and α rRNA species (not shown). These results indicate that the slower bands represent rRNA aggregates. The small-size bands might be analogous to the 5.8 S and 5 S species commonly found in mammalian cells. Nevertheless, when the same rRNA preparations were fractionated in a more discriminating system (on short polyacrylamide denaturing gels) for the region occupied by the two small-size bands, these two bands were resolved into five discrete bands without smearing material (Fig. 1b); in addition to a broader band which migrated in the zone of mouse transfer RNA. This finding was not observed with mouse liver rRNA which had not been especially protected from RNAase activity as described in Methods (not shown). Therefore, the initial two small-size bands, pseudo 5.8 S and pseudo 5 S, actually represent an heterogeneous assembly of multiple molecules of definite sizes.

The small-size rRNA species were found complexed, after RNA extraction and denaturation, in the large ribosomal subunit which was obtained by fractionation of ribosomes on isokinetic sucrose gradients with high K^+ concentration (not shown). This large-subunit association has been reported also for *C. fasciculata* [12]. The small-size species might bind, as a gross approximation, ribosomal proteins or rRNA. We explored this possibility by the use of deproteinized rRNA or deproteinized and denatured rRNA. The deproteinized material of the agarose pseudo bands (Fig. 1A) was independently electroeluted, heat denatured in the presence of 7 M urea, and

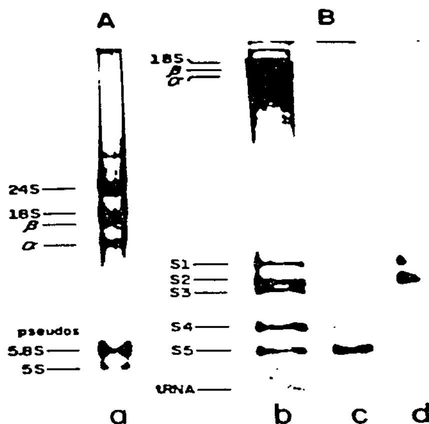

Fig. 1. Photographs of *T. cruzi* rRNA separated on gel electrophoresis. The rRNA preparations were applied to long nondenaturing (A) and short denaturing (B) slab gels (see Materials and Methods). Tracks a, b: total rRNA; track c, pseudo 5 S, track d pseudo 5.8 S.

reelectrophoresed on denaturing polyacrylamide gels. The pseudo 5.8 S material yielded the two uppermost bands (Fig. 1d) and the pseudo 5 S, the two lowest bands (Fig. 1c) of the group of five small-size species (thereafter called and numbered s1 to s5 beginning with the largest). Since the s3 species was not found in the pseudo 5.8 S and 5 S materials obtained from deproteinized but nondenatured total rRNA, the binding of this species is apparently not dependent on proteins as is the binding of the other species. It should, then, be primarily hydrogen bonded to rRNA. We tested this assumption by two kinds of experiments.

Deproteinized total rRNA was fractionated on fully nondenaturing sucrose gradients [1]. Fractions of the three main peaks in the regions of 0–6 S, 18 S and 24 S (containing the α and β species) were pooled (Fig. 2A, brackets), ethanol precipitated, and redissolved in the denaturing electrophoresis solution. The totality of the material obtained from each main peak was independently heated and layered onto denaturing gel slabs. The 0–6 region of rRNA contained the s1, s2, s4 and s5 species (Fig. 2a). The 18 S rRNA did not contain any small size species, except a very light and contaminating s3 material barely seen in the photograph (Fig. 2b). Finally, the 24 S rRNA

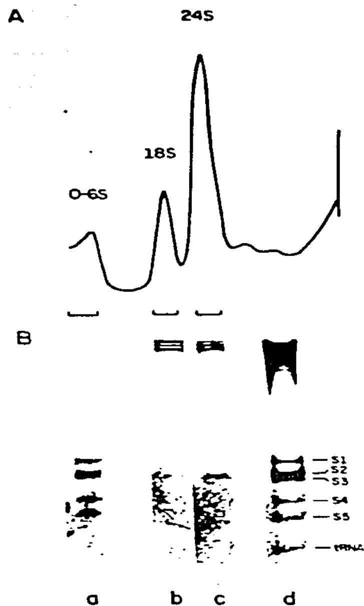

Fig. 2. Fractionation of *T. cruzi* rRNA on nondenaturing isokinetic sucrose gradients (A), and on denaturing slab gels (B). All the material from the pooled gradient fractions, shown in brackets, was applied to denaturing slab gels. Track a: 0–6 S rRNA, track b: 18 S rRNA; track c: 24 S rRNA; track d: total rRNA layered onto the sucrose gradient.

revealed, upon denaturation, the missing s3 species (Fig. 2c). Increased amounts of loaded material on tracks a, b and c did not produce qualitative changes in the gel patterns.

To determine if the s3 species is bound to the α or β species, deproteinized total rRNA was electrophoresed on nondenaturing agarose gels (as in Fig. 1A). Material from the α and β species, and 24 S rRNA was eluted and reelectrophoresed on denaturing polyacrylamide gels (as in Fig. 1B). The α species did not release any

TABLE I

Size values of trypanosomatid small-size rRNA species

rRNA species	Number of nucleotides		
	T. cruzi[a]	*T. brucei*[b]	*C. fasciculata*[c]
s1	261 ± 3	215	238
s2	217 ± 3	190	193
s3	197 ± 3	180	176
s4	141 ± 2	125	135
s5	110 ± 2	115	120

[a] Figures represent the mean ± S.E.M. from five individual gels. The values for the size standards mouse 5 S and 5.8 S, and *T. cruzi* α rRNA species are 121, 159 [13], and 1 661 [1] nucleotides, respectively. The line representing the least squares fit was $L = 2276.7 \cdot 1.386^{t}$ (correlation coefficient $r = 0.9999$).

[b] Data from Cordingley and Turner [11]

[c] Data from Gray [12]

material. The β species, and of course the 24 S rRNA (containing both α and β), showed the s3 species (gels not shown).

Molecular sizes of the five small-size rRNA species were calculated in the polyacrylamide-urea gel system by the least squares analysis of the logarithm of the number of nucleotides versus the migration distance at band center, using mouse 5 S and 5.8 S and *T. cruzi* α rRNA species as size markers. The median size values of *T. cruzi* small-size rRNA species were found to be very similar to those reported for *T. brucei* [11] and *C. fasciculata* [12], as can be seen in Table I. When the A_{260} values of the fractionated large- and small-size rRNA species, obtained from a same total rRNA preparation, were normalized against their corresponding molecular sizes, the stoichiometry of all eight molecules in relation to each other was about 1 (calculations not shown).

DISCUSSION

The mature cytoplasmic rRNA species of trypanosomatids seem to be unique among both prokaryotes and eukaryotes. To date, this distinction appears at least in two rRNA characters: the size of the 18 S species and the total number of species. These differences are important in view of the high conservation of these parameters along phylogeny. The known molecular masses of the 18 S rRNA of different biological species can be assembled into two groups of approximately 0.5 and 0.7 × 10^6 daltons in prokaryotes and eukaryotes, respectively. *T. cruzi* 18 S rRNA has a molecular mass of about 0.84 × 10^6 daltons; therefore, it falls in a third group. Other trypanosomatids and protozoa also show this high value for the 18 S rRNA; the

validity of these data has already been discussed [1]. Furthermore, *T. cruzi* and other organisms present three large-size species (see Introduction).

The small-size rRNA molecules in most eukaryotes are the 5.8 S and the 5 S. Our previous assumption that the two *T. cruzi* light bands seen in agarose gels (Fig. 1A) corresponded to these two small-size species [1] was false. The pseudo 5.8 S material actually contains three species: s1, s2 and, under denaturing conditions, s3; and the pseudo 5 S contains two species: s4 and s5. That is, this organism has five small-size species instead of two. These five species, most probably are natural and not due to nonspecific degradation since: (1) they were obtained under conditions of nondetectable RNAase activity [1]; (2) the 18 S, and the α and β species (Figs. 1 and 2) did not show degradation;(3) these multiple species were not found in mammalian ribosomes (obtention of mouse 5.8 S and 5 S for experiments of Table 1); (4) they were present in the same number and proportions under different isolation procedures; and (5) they have been found in two other trypanosomatids [11,12]. *Leishmania tarentolae* also contains the eight small- and large-size rRNA species; but the 'extra' large-size rRNA visualized in sucrose gradients and on polyacrylamide gels was interpreted as a breakdown product, and three of the small-size species as the commonly known 4 S, 5 S and 5.8 S [14].

The overall association behavior of the trypanosomatid small-size rRNA species is, nevertheless, similar to that shown by the 5.8 S and 5 S species: (1) all are bound to the large ribosomal subunit; (2) the s1, s2, s4 and s5 are probably bound to ribosomal proteins (they are released by low Mg^{2+} concentrations, sodium dodecyl sulfate, and/or deproteinization) like the 5 S species; and (3) the s3 is hydrogen bonded to the β species of the 24 S rRNA (it is only released by RNA denaturation) like the 5.8 S to the 28 S species; although s3 is larger than 5.8 S (Table 1).

Initial RNA:DNA hybridization experiments with *T. cruzi* material (see following paper) [15] indicate that s1, s2 and s3 are physically linked to the rRNA cistrons. These three species are thus putative products of the rRNA primary transcript processing. The hydrogen bonding of s3, equivalent to that of 5.8 S whose DNA sequence lies in the transcribed spacer of the rRNA cistron, further supports this idea. The s1 and/or s2 might be similar to *D. melanogaster* 2 S which is a maturation product of the precursor 26 S. Although 2 S, unlike s1 and s2, is only 30 nucleotides long and is hydrogen bonded to the mature 26 S [16]. *T. cruzi* s4 and s5, unlike the *C. fasciculata* small-size species [12], do not hybridize in the rRNA cistron; they might, then, resemble mammalian 5 S which also maps outside the rRNA cistron and appears to bind ribosomal proteins. The implications of these data are still unknown, but they might be related to some fine differences between trypanosomatidian and mammalian protein synthesis, and to the phylogeny of lower eukaryotes. Actually, the 5 S rRNA of *Euglena gracilis* is more closely homologous to the corresponding s5 species (in our nomenclature) of *C. fasciculata* than it is to the 5 S of plants and green algae [17].

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AN ENDONUCLEASE RESTRICTION ANALYSIS OF THE RIBOSOMAL RNA GENES OF *TRYPANOSOMA CRUZI*

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The location of ribosomal RNA genes in total-nuclear-enriched DNA preparations of *Trypanosoma cruzi* was analyzed by using restriction endonucleases and the eight cytoplasmic ribosomal RNA species of this organism. Two contiguous *SstI* DNA fragments of about 9.88 and 1.7 kilobase pairs contained the three large-size ribosomal RNA species, 18 S, β, and α, and three small-size ribosomal RNA species, s1, s2 and s3. The other two small-size ribosomal species, s4 and s5, were located outside the ribosomal RNA cistron and independently of each other. Spacers of a presumed large length, about 20 kilobase pairs or more, hampered the identification of putative adjacent ribosomal RNA cistrons.

Key words: *Trypanosoma cruzi*; Ribosomal RNA genes, Restriction endonuclease mapping.

INTRODUCTION

The number and the structural organization of genes coding for ribosomal RNA (rRNA) have been studied in widely different organisms [1]. These genes are present in a varied number of repeating units, from a few in prokaryotes [2,3], in eukaryotic cell organelles [4], and in micronuclei of a ciliated protozoan [5], to up to thousands of amplified and extrachromosomal genes in oocytes of many metazoa [6,7]. In the 3'-5' direction of the coding DNA strand, each genetic unit contains sequence information for the eukaryotic 18 S, 5.8 S, and 28 S (common nominal values) rRNA species, which is followed by a spacer region containing some small stretches of abortive transcription [8]. In some organisms such as *Escherichia coli* [2], *Saccharomyces cerevisiae* [9], *Dictyostelium discoideum* and probably *D. mucoroides* and *Polysphondylium violaceum* [10], and *Euglena gracilis* [11], the cistron for the 5 S species is linked to the rRNA cistron. The repeating genetic units of rRNA are clustered in the genome with the

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Abbreviation: kb, kilobases or kilobase pairs.

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exception of those present in the macronucleus of *Oxytricha fallax* [12]. The units in the cluster, arranged in the same 18 S to 28 S direction, either are in tandem as in higher eukaryotes [13,14] and in some lower eukaryotes [11,15–17], or are in palindromic dimers as in other lower eukaryotes, with an axis of symmetry near either the 18 S [10,18,19] or the 28 S end [20].

Trypanosoma cruzi contains some 114 nuclear rRNA genes and this amount agrees well with those of other organisms in relation to their position in phylogeny [21]. However, the number of cytoplasmic rRNA species of *T. cruzi* differs greatly from those of other organisms, since *T. cruzi* has three large-size species (18 S, α and β) [21] and five small-size species (s1 to s5) [22]. The *T. cruzi* α and β species compose the 24 S rRNA; similar rRNA species are present also in other trypanosomatids, in other protozoa, in protostomia, and in *Drosophila melanogaster*, cited in preceding paper [22]. In *D. melanogaster* [23] and *Leishmania donovani* [17] both rRNA species map in the nominal 28 S rRNA gene and, therefore, might have a role in the mechanism of protein synthesis similar to that of the 28 S rRNA species in mammals. To date, the five small-size rRNA species seem to be general to all trypanosomatids (see ref. 22), but restricted to this group of organisms; there is no information regarding their functional role or their individual arrangement in the rRNA cistron. Here, we report on an analysis of the organization of the *T. cruzi* rRNA genes, using restriction endonucleases and the eight rRNA species as hybridization probes.

MATERIALS AND METHODS

Growth of cells. The *T. cruzi* isolate and the procedures for its culture and collection have been described in the preceding report [22].

Isolation of total-nuclear-enriched DNA. T. cruzi cells were harvested by centrifugation at 10 000 × *g* for 10 min at 4°C and then were washed twice with 30 mM Tris-HCl (pH 7.8), 100 mM KCl, 5 mM $MgCl_2$, 3 mM $CaCl_2$. Cells were resuspended in the same solution and were lysed by the addition of 1% Nonidet P-40 (final concentration). The lysate was centrifuged at 10 000 × *g* for 20 min and the pellet was resuspended in 150 mM NaCl, 10 mM EDTA, 15 mM trisodium citrate. Under these conditions, most of the kinetoplasts remained in the supernatant as determined by phase microscopy. The resuspended pellet was incubated for 2 h at 37°C in the presence of 1 mg ml^{-1} of pronase preincubated under the same conditions. After the digestion had been terminated by the addition of 1 M $NaClO_4$ (final concentration), the material was emulsified with 2 volumes of chloroform-isopentanol (24:1). Further treatment of the DNA with pancreatic ribonuclease and the same solvent system was done as previously described [21].

Isolation, fractionation, and labelling of rRNA. The isolation and fractionation procedures were basically as in the preceding report [22]; the large-size species were

separated on the agarose gels and the small-size species on the polyacrylamide gels. After being stained with ethidium bromide (3µg ml^{-1}), the rRNA bands were cut out and electroeluted. The bands corresponding to species 18 S, β, s2 and s3 were further purified from contaminating material from adjacent bands by a second electrophoresis. Since the other four rRNA species showed good resolution in the first fractionation [22], this last procedure was unnecessary for them.

The individual rRNA species isolated by electrophoresis were radioactively labelled by phosphorylation in vitro with T4 polynucleotide kinase (Bethesda Research Laboratories, Inc.) and [γ-^{32}P]ATP (New England Nuclear). RNA was first pretreated to increase the proportion of free 5'-hydroxyls: (a) large-size species were partially hydrolyzed in 0.025 ml of 25 mM Tris-HCl (pH 9.5) at 90°C for 30 min, and (b) small-size species were dephosphorylated with bacterial alkaline phosphatase (20 units/0.05 ml) in 10 mM Tris-HCl (pH 8.0) at 37°C for 1 h, reextracted with phenol-chloroform (1:1), and then precipitated with ethanol after prior addition of 0.8 M LiCl (final concentration). The labelling reaction mixture (0.06 ml total volume) containing 10 mM Tris-HCl (pH 9.5), 10 mM $MgCl_2$, 10 mM spermine, 10 mM dithiothreitol, 0.5–1.0 µg RNA, 10 units T4 kinase, 200 µCi [γ-^{32}P]ATP was incubated at 37°C for 1 h. The reaction was terminated by the sequential addition of 0.18 ml of 8 M LiCl, 1 M ammonium acetate, 2.5 volumes of ethanol, and 500 µg of yeast soluble RNA. The precipitate was collected by centrifugation and redissolved in 0.6 ml of 100 mM EDTA (pH 7.4), 20% glycerol, 1% Sarkosyl. The mixture was applied to a Sephadex G-25 column (1 × 15 cm) and eluted with 10 mM Tris-HCl (pH 7.0), 100 mM NaCl, 10 mM EDTA. The fractions containing the labelled rRNA were pooled and the material was precipitated as described above. The specific activity of the rRNA was 10^6–10^7 cpm µg^{-1}.

Restriction endonuclease digestion and gel electrophoresis of DNA. All restriction endonucleases were titrated and used as recommended by the supplier (Bethesda Research Laboratories, Inc.). The reaction mixtures (0.02 ml total volume) with *T. cruzi* DNA contained a 5 to 10 fold excess of endonuclease in relation to phage λ DNA which was used as internal limit digestion control. The reactions were terminated by the addition of 2 M urea, 0.05% bromophenol blue (final concentrations). The digested DNA was then subjected to electrophoresis on 0.8% agarose slab gels (20 cm length) as described by Firtel et al. [24]. Phage λ DNA digested with *Hind*III was used as size standard [25].

RNA:DNA hybridization on Southern imprints. Restricted DNA was eluted from the agarose gel slabs onto nitrocellulose sheets according to Southern [26]. Radioactively labelled rRNA (1–5 × 10^6 cpm) was hybridized to the DNA imprints in 10 ml of hybridization solution, 120 mM phosphates (pH 7.0), 150 mM NaCl, 15 mM trisodium citrate, 10 mM EDTA, 50% formamide, 0.2% sodium dodecyl sulfate for 24 h at 37°C. In experiments using the β species, due to partial contamination, a 20 fold excess

of both unlabelled 18 S and α species were used to compete with it. After hybridization, the nitrocellulose sheets were rinsed with 100 ml of 300 mM NaCl, 30 mM trisodium citrate, and then washed four times with 25 ml of the hybridization solution at 37°C for at least 4 h each, with rinsings in between. Autoradiographs were prepared by exposing the nitrocellulose sheets to X-ray films (Kodak X Omat RP) at −70°C in the presence of intensifier screens.

RESULTS AND DISCUSSION

Restriction endonuclease analysis is facilitated in DNA molecules of reduced sequence complexity. Although *T. cruzi* nuclear DNA does not show density satellites in neutral CsCl, we tried to obtain enriched preparations of rRNA genes by fractionation in preparative CsCl-actinomycin D gradients. Radioactively labelled rRNA hybridized mostly to the main peak of DNA which was positioned at about 1.606 g ml^{-1} and only slightly to a small peak of DNA positioned at about 1.642 g ml^{-1}. The analyzed range of density was 1.7 to 1.5 g ml^{-1} (unpublished experiments). Therefore, the restriction analysis was done with the use of total-nuclear-enriched DNA.

We tested 11 restriction endonucleases (*Bam*HI, *Eco*RI, *Hae*II, *Hha*I, *Hind*III, *Kpa*I, *Sal*I, *Sma*I, *Sst*I, *Xba*I and *Xho*I). Electrophoresis gels of the resulting digests were intially visualized by using ethidium bromide and UV light. DNA fragments appeared as a smear along the tracks with some discrete bands. In all digests, there were two bands of about 1–1.5 kilobase pairs (kb) which were probably of kinetoplast origin [25] and which did not show any rRNA hybridization signal (see below). Only two of the above enzymes, *Hind*III and *Sst*I, produced digests containing strong rRNA hybridization bands that were smaller than the bulk DNA (ca. 20–25 kb) and therefore were amenable for subsequent analysis.

To position the genes for cytoplasmic rRNA on the restriction endonuclease map of nuclear DNA, the eight *T. cruzi* cytoplasmic rRNA species were individually isolated and radioactively labelled in vitro. Each species was hybridized to DNA imprints of fragments generated by *Hind*III and/or *Sst*I. Fig. 1 shows the hybridization of the three ^{32}P-labelled large-size species; Figs. 2 and 3, that of the small-size s1 to s3, and s4, s5 species, respectively. Table I summarizes these data and shows that DNA sequences containing rRNA complements could be divided into three linkage groups, one for the majority of the rRNA species and two other groups with one rRNA species each: (1) DNA fragments 1 to 6 hybridized to the three large-size species (18 S, α and β) and to small-size species s1, s2 and s3; (2) DNA fragments 7 to 10 hybridized to s4; and (3) DNA fragment 11 (which could be composed of several fragments because of the low resolution of these sizes in the agarose gels) hybridized to s5. This grouping indicates that six rRNA species are represented in the rRNA cistron and that the other two species have their corresponding genes outside the rRNA cistron.

Since, as shown in Fig. 1, DNA fragment 2 (9.88 kb) disappeared in the double digestion with a simultaneous production of fragments 3 (5.6 kb) and 4 (3.74 kb) and

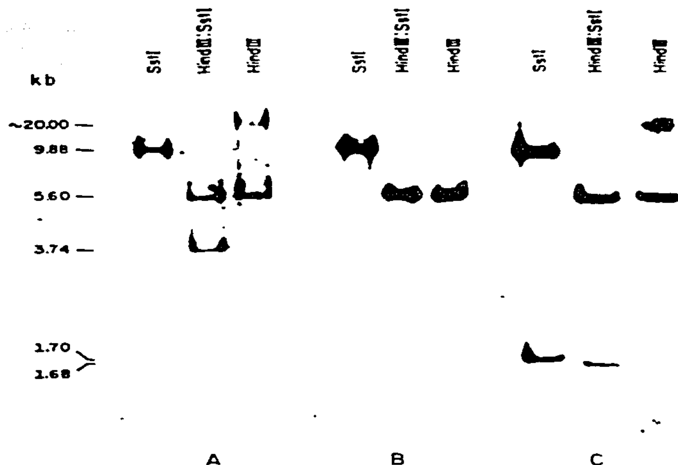

Fig. 1. Hybridization of ^{32}P-labelled large-size rRNA species to restriction endonuclease fragments of *T. cruzi* DNA. Total-nuclear-enriched DNA preparations (see Methods) were digested with restriction endonucleases. The DNA fragments were separated by electrophoresis on 0.8% agarose slab gels. The ^{32}P-labelled rRNA was hybridized to DNA imprints of the fragments, and the resulting hybrids were detected by autoradiography. Fragment sizes shown are for the hybrid bands; *Hind*III digests of λ DNA were used as size standards [25]. (A) 18 S rRNA, (B) β rRNA, and (C) α rRNA.

since all three have common hybridizing rRNA species (Table I), these smaller DNA fragments are contained in fragment 2. The difference in size, about 0.54 kb, between fragment 2 (9.88 kb) and the sum of fragments 3 and 4 (9.34 kb) may be accounted for by the existence of silent sequences, since no hybridizing material larger than 0.2 kb was found in DNA digests resolved on 1.2% agarose and with overexposed transfer blots (not shown). Or, this difference could simply be an overestimation of the 9.88 kb value, since migration in agarose gels is not linear for these large-size molecules. Using similar reasoning, we concluded that DNA fragment 5 (1.7 kb) contains fragment 6 (1.68 kb).

The DNA fragments comprising the first linkage group can thus be arranged in two

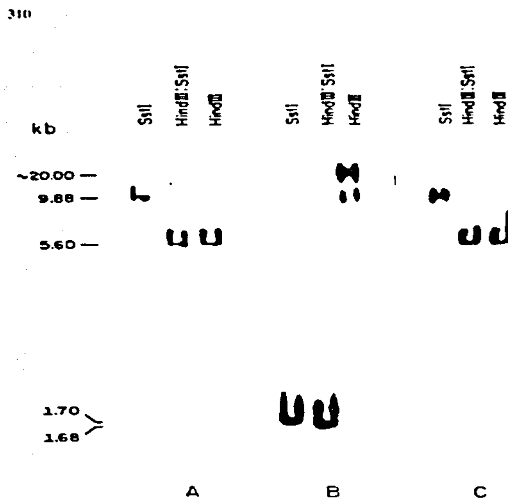

Fig. 2. Hybridization of ^{32}P-labelled small-size rRNA species to restriction endonuclease fragments of *T. cruzi* DNA. (A) s1 rRNA; (B) s2 rRNA; and (C) s3 rRNA. Details as in Fig. 1.

possible linear relationships (Figs. 4A and 4B). Since the α species hybridized to DNA fragments 3, 5, and 6 (5.6, 1.7, and 1.68 kb, respectively); the 18 S species to fragment 4 (3.74 kb); and a DNA fragment of about 3.76 kb is not found in the single *Hind*III digestion, the model in Fig. 4B is the correct relationship. In addition, fragments 2 and 3 (9.88 and 5.6 kb) contained sequences complementary to five (their added size, about 6.66 kb) of the common six rRNA species, with the exception of s2. This further indicates that the genes for these rRNA species are clustered. Although species s2 did not hybridize with fragments 2 or 3, it did hybridize to fragments 5 and 6 (1.7 and 1.68 kb) which are adjacent to fragment 3 (5.6 kb) (Fig. 4B); therefore, it is also clustered.

The spatial arrangement of the genes for the rRNA species was constructed by using: (1) the lengths of the cytoplasmic rRNA species (about 2.5, 2.0 and 1.7, and 0.26, 0.22, 0.20, 0.14 and 0.11 kb for the 18 S, β and α, and s1 to s5 species, respectively) [21,22]; and (2) the relative intensity of hybridization of these eight probes to the various DNA fragments. Therefore, the positions of the termini of the cytoplasmic rRNA genes depicted on the restriction endonuclease map shown in Fig. 5 are only

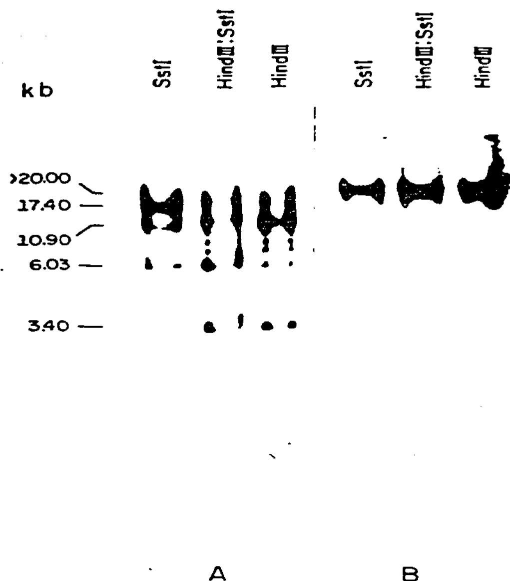

Fig. 3. Hybridization of ³²P-labelled small-size rRNA species to restriction endonuclease fragments of *T. cruzi* DNA. (A) s4 rRNA; and (B) s5 rRNA. Details as in Fig. 1.

tentative. Likewise, the position for the s1 and s3 genes, which have the same hybridization pattern (Table I), are only grossly approximated and we lack data on their relative order. The 3'-5' direction has been chosen only by analogy to the known rRNA cistrons.

The spacer regions outside the rRNA cistron most probably present length or sequence heterogeneity. This is indicated by the presence of light bands of hybridization in *Hind*III digests with: (1) the 18 S species at about 10, 8 and 7 kb upstream of the *Hind*III site in the 18 S gene (Figs. 1A and 5), in addition to the strong band at about 20

TABLE 1

Main restriction endonuclease fragments of total-nuclear-enriched DNA from *T. cruzi* and their hybridization to rRNA.

DNA fragments			Cytoplasmic rRNA species							
No.	kb	Restriction enzymes	18S	β	α	s1	s2	s3	s4	s5
1	20.00	*Hind*III	+	-	+	-	+	-	-	-
2	9.88	*Sst*I	+	+	+	+	-	+	-	-
3	5.60	*Hind*III; *Hind*III: *Sst*I	+	+	+	+	-	+	-	-
4	3.74	*Hind*III: *Sst*I	+	-	-	-	-	-	-	-
5	1.70	*Sst*I	-	-	+	-	+	-	-	-
6	1.68	*Hind*III: *Sst*I	-	-	+	-	+	-	-	-
7	17.40	*Sst*I	-	-	-	-	-	-	+	-
8	10.90	*Hind*III: *Sst*I	-	-	-	-	-	-	+	-
9	6.03	*Hind*III; *Sst*I; *Hind*III: *Sst*I	-	-	-	-	-	-	+	-
10	3.40	*Hind*III; *Hind*III. *Sst*I	-	-	-	-	-	-	+	-
11	>20.00	*Hind*III: *Sst*I; *Hind*III: *Sst*I	-	-	-	-	-	-	-	+

kb; and (2) the α and s2 species at about 10 kb downstream of the *Hind*III site in the α gene (Figs. 1C and 5), in addition to the strong band at about 20 kb. Although the presence of the large spacers, approximately 20 kb to either side of the rRNA cistron (same Figs. 1A, 1C and 5 and Table 1, fragment 1), have precluded us from locating the

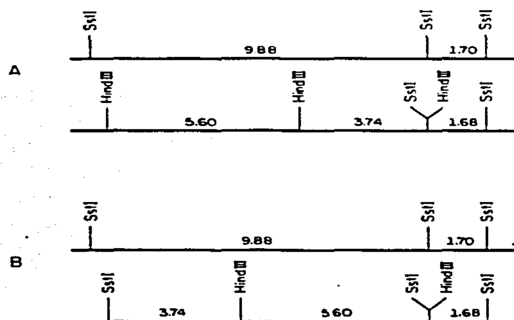

Fig. 4. Two possible arrangements of the members of the first linkage group of *T. cruzi* restriction endonuclease DNA fragments. Fragment values in kb.

Fig. 5. Restriction map of the *T. cruzi* rRNA cistron. The unit is contained on two adjacent *Sal*I DNA fragments, 9.88 and 1.7 kb. Dashed lines indicate probable infrequent *Hind*III sites. The 3'-5' direction is from left to right.

next rRNA cistron, the simplicity of the patterns of hybridization with the six linked rRNA species indicates that the cistrons are probably contiguous to one another. Nevertheless, the precise length of these spacers is difficult to confirm since we lack probes and information on additional restriction endonuclease sites along this length.

The genes for the s4 and s5 species, unlike those in *Crithidia fasciculata* [27], are not in the rRNA cistron (Table I), and are each in different regions of the genome since they show different hybridization patterns (Fig. 3). The genes for the s4 species are located in multiple different sites (Fig. 3A, Table I). Those for the s5 species are probably also multiple because of the great width of the hybridization bands (Fig. 3B), but we do not have data to determine if they are clustered or interdispersed.

The gross structural features of the *T. cruzi* rRNA cistron are very similar to those of *L. donovani* [17] and other eukaryotes in the mutual arrangement of the large-size rRNA species, and in that small-size species map between the two common large-size species; s3 being the biochemical equivalent of 5.8 S due to its hydrogen bonding to the β species [22]. Nevertheless, the *T. cruzi* rRNA cistron shows some peculiarities: (1) Its 18 S rRNA gene does not present an *Eco*RI site (data not shown) near the 5' end as most eukaryotes do in their corresponding genes. Although this restriction site is also absent in some other eukaryotes such as *Tetrahymena pyriformis*, cited in [14], *Physarum polycephalum* [19], *Neurospora crassa* [28], *Caenorhabditis elegans* [29], *Artemia* larvae [30], and some cereals [31]. (2) It contains three small-size rRNA species (s1 to s3). The position of s2 near the 3' end of the α rRNA species, which is located at about the distal half of the equivalent 28 S species, is interesting in view of the known lack of precursor rRNA processing at this terminus. On the other hand, the s2 gene may be equivalent to a linked 5 S gene; although s4 and s5 are also putative equivalent 5 S species due to their hybridization outside the rRNA cistron. More detailed sequence information on these molecules and precursor rRNA will be needed to resolve this problem, and to determine more precisely the length of the flanking spacer regions.

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OBJETIVO

Dada la situación de que los ribosomas citoplásmicos de *T. cruzi* presentan moléculas adicionales de RNA, y de que la mayoría de dichas moléculas hibridan en la unidad fundamental de transcripción; los objetivos de esta Tesis son los siguientes:

1. La construcción de bibliotecas genómicas de *T. cruzi*, y el aislamiento de clonas recombinantes con DNA ribosomal.

2. La localización de las secuencias de DNA codificadoras para las moléculas pequeñas de RNA ribosomal, con respecto a la ubicación génica de las moléculas mayores.

TRABAJO REALIZADO

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Molecular cloning and partial characterization of ribosomal RNA genes from *Trypanosoma cruzi*

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To further analyze the organization of the nuclear rDNA locus in *Trypanosoma cruzi*, genomic recombinant plasmid clones were constructed and isolated after hybridization with rRNA molecules as hybridization probes. Approximately 11 kilobase pairs from the cistron were cloned in three recombinant plasmids carrying adjacent genomic fragments. Restriction mapping and Southern hybridization experiments performed on these clones indicate the following relative arrangement of the mature rRNA coding sequences: 18S (2.46 kb), S3 (197 b), 24Sα (2.02 kb), S1 (261 b), 24β (1.66 kb), S2 (217 b) and S6 (90 b). Neither S4 (141 b) nor S5 (110 b) sequences were found within these genomic clones. Nevertheless genomic Southerns suggest a linkage of S4 towards the 3' end of this genetic system.

Key words: *Trypanosoma cruzi* rDNA; Plasmid molecular cloning; Restriction mapping

Introduction

To date, Trypanosomatids are the group of organisms which exhibit the most complex pattern of mature cytosolic rRNA molecules [1–3]. The 24S molecular mass species is composed of two similar sized independent molecules α and β, which under non-denaturing conditions are hydrogen bonded [4]. Our previous size correspondence of 24Sα and 24Sβ is here changed to 2.02 and 1.66 (kb) kilobase pairs respectively to unify nomenclature with the equivalent molecules described in *Drosophila* [5] and *Trypanosoma brucei* [6].

Although a hidden break has been described in organisms from different phyla like Protozoa, Mollusca, Annelida and Arthropoda [3,4,7], the presence in Trypanosomatids of multiple small-sized RNAs [1–3] is quite different from any other phylogenetic group observed to date. Their cytosolic ribosomes contain equimolar amounts of six small rRNA molecules (S1–S6) [1]. The smallest one (S6) has an electrophoretic mobility similar to that of mammalian tRNA species [2,3] and was initially considered a putative tRNA. Sequence analysis of S3 and S5 [8–10] demonstrates their correspondence to the 5.8S and 5S rRNAs of other eukaryotes.

In *Trypanosoma cruzi*, most of these small rRNAs map within the main transcription unit [11], and their relative position with respect to the large molecular mass species has been documented in African trypanosomes [6,12]. Our previous map of this locus in *T. cruzi*, based on genomic Southern hybridizations [11] is here extended with the construction, isolation and partial characterization of genomic plasmid clones. The analysis reported in the present work led to the relocation of S1 and the inclusion of S6. The genomic mapping of S4 was also reconsidered. Our data show an rRNA coding sequence organization similar to that recently reported for the Trypanosomatids *T. brucei* [6] and *Crithidia fasciculata* [13].

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Abbrevations: kb, kilobase or kilobase pairs; (S1–S6), small-size ribosomal RNAs.

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Materials and Methods

Cell culture and nucleic acids preparation. Epimastigotes from a Mexican stock of *T. cruzi* (isolated in la Cruz, Jalisco, Mexico by Dr. J. Tay, School of Medicine, UNAM), were axenically cultured as described elsewhere [4]. Isolation of both nuclear enriched DNA, and ribosomal RNA were performed as previously described [3,11].

Cloning and characterization of T. cruzi ribosomal DNA. T. cruzi genomic DNA was digested with restriction enzymes, ligated into plasmid pUC18 [14], and transformed into *Escherichia coli* MC1061 [15] as described by Maniatis [16]. Colonies were screened as described by Rowekamp and Firtel [17] using kinase end-labelled rRNAs [11]. Plasmids were purified on $CsCl_2$/ethidium bromide gradients by standard procedures [16]. Partial endonuclease digestions for restriction site mapping were performed as described by Smith and Birnsteil [18] using end-labeled DNA molecules prepared as described elsewhere [19]. Southern blot experiments of digested clones of genomic DNA were performed by standard techniques [20]. RNA blots were obtained by two different procedures: (a) large molecules (18S, 24Sα, and 24Sβ) were Northern transferred from 6% formaldehyde/1% agarose gels [21] to Gene Screen membranes (New England Nuclear) according to the manufacturer's directions; (b) small size rRNA molecules (S1–S6) were first individually electroeluted [11], denatured and vacuum filtered onto nitrocellulose sheets using a Bio-Rad dot blot apparatus. After being air dried all filter membranes were vacuum baked at 65°C for 90 min.

Prehybridization and hybridization conditions for DNA:DNA renaturation were 50% formamide/300 mM NaCl/30 mM trisodium citrate/120 mM phosphates (pH 7.0)/4 × Denhardt's solution [22]/2 mM EDTA (pH 7.2)/0.2% sodium dodecyl sulfate at 37°C for 2 and 24 h, respectively. DNA:RNA hybridization conditions were identical to the above, except that half of NaCl and of trisodium citrate were used. Washing conditions for both kinds of hybridizations were: 15 mM NaCl/1.5 mM trisodium citrate/0.2% sodium dodecyl sulfate for 2 h at 37°C. Filters were exposed to X-ray films with intensifier screens at −70°C.

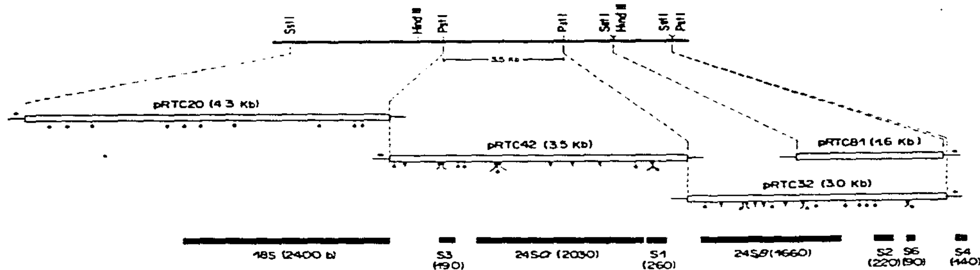

Fig. 1. Map of the ribosomal RNA cistron in *T. cruzi*. Genomic DNA restriction fragments (upper line) were inserted into the *Pst*I and/or *Sst*I sites of pUC18. Open bars indicate cloned genomic inserts, whereas flanking lines are vector sequences. To show the polarity of the insert in the vector, the *Eco*RI site within the pUC18 polylinker is indicated as *. The restriction cleavage sites mapped in the cloned inserts, besides those indicated in the genomic DNA are: ↓ *Hinc*II, ↓*Sau*3A and ↑ *Hinf*I. The latter sites have not been analysed in clone pRTC20. Restriction sites in clone pRTC81 are not drawn, but correspond to those indicated in the overlapping region of clone pRTC32. The location of the mature rRNA species (closed bars) is shown below those restriction fragments that exhibited hybridization signal in autoradiographs of Southern blots. The exact position of the 5′ and 3′ ends cannot be determined from this kind of data. S4 sequences were not cloned. Its genomic hybridization pattern (Fig. 3) suggests the linkage here shown.

Results and Discussion

Cloning strategy, screening and classification of clones. Our previous rRNA genomic map based upon *Hin*dIII and *Sst*I restriction sites [11] was extended by the location of three *Pst*I sites (Fig. 1 upper line). We decided to clone *T. cruzi* genomic DNA fragments using the *Pst*I and *Sst*I cloning sites of the plasmid vector pUC18 [14] (see Material and Methods). Ribosomal DNA recombinant bacterial clones were isolated from these genomic libraries by screening the cells with a radioactive mixture of rRNA molecules. About 20 positive clones were identified and further classified in 4 groups according to the following criteria: (a) restriction site(s) ligated in the construction, (b) DNA insert size cloned, and (c) hybridization to the different large and small rRNA species. The recombinant plasmid clones pRTC20, pRTC42, pRTC32 and pRTC81 represent the 4 different types of ribosomal DNA fragments cloned (Fig. 1). The different rRNA coding sequences present within these clones were determined by hybridization to the 3 large rRNA species (18S, 24Sα and 24Sβ) separated on agarose/formaldehyde gels (Fig. 2A), and to the 6

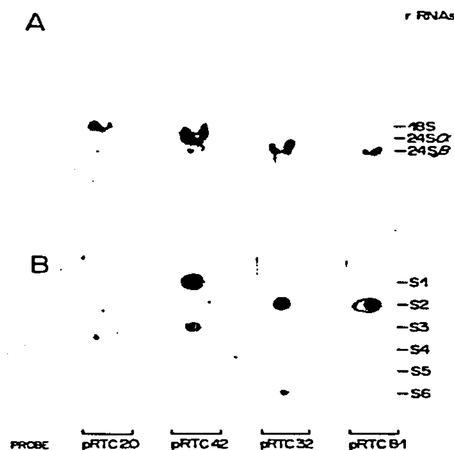

Fig. 2. Hybridization patterns of the recombinant clones. Large rRNA molecules were resolved on agarose/formaldehyde gel electrophoresis [21] and transferred to Gene Screen membranes (A). Small rRNAs were individually electroeluted from polyacrylamide/urea gels and dot blotted unto nitrocelulose sheets (B). Both membranes (A and B) were hybridized with nick-translated clones pRTC20, pRTC42, pRTC32 and pRTC81. Positions of 18S, 24Sα and 24Sβ rRNA molecules (A) were identified after methylene blue staining of the filter strips [16].

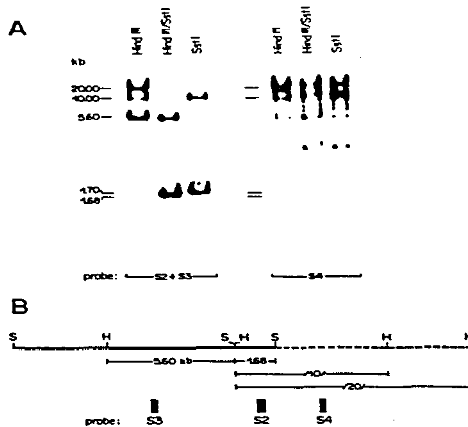

Fig. 3. Hybridization of small size rRNA species to restriction fragments of *T. cruzi* genomic DNA. Both hybridizations shown were carried out from the same slab gel. Fragment sizes are approximate and were estimated using λ *Hin*dIII digests as size markers (A). For the recognition of S2 and S3 hybridizing fragment within the map (B), see Fig. 1. S4 fine location is unknown, but the above data shows that it may be downstream the last *Sst*I site cloned (dark line). Discontinuous line indicates an apparently heterogeneous region which contains S4 related sequences. The multiple *Sst*I sites within this region are not drawn. Finally, the weak hybridization signal of the 5.60 kb *Hin*dIII fragment seen with S4 as a probe may be due to a cross hybridization as described elsewhere [12].

small size rRNA molecules (S1–S6) electroeluted from gels and individually dot-blotted onto nitrocellulose sheets (Fig. 2B).

None of the clones hybridized to S4 or to S5; nevertheless genomic mapping experiments are consistent with a linkage of S4 sequences within large *Hin*dIII fragments, downstream the last 3′ *Sst*I site cloned in pRTC81 (Fig. 3). This DNA region appears to be heterogeneous in length and/or sequence, and the putative linkage of S4 will be confirmed through chromosome walking cloning experiments. Mapping of S4 towards the 3′ end of the rRNA cistron has been documented in *T. brucei* [6] and in *C. fasciculata* [13].

Restriction site mapping and Southern hybridizations. In order to map the coding regions of the small rRNAs with respect to those of the large molecular-mass species, inserts from the genomic clones were further characterized by restriction site mapping. The cut sites for some endonucleases were located in the cloned inserts using partially digested end-labelled molecules (Fig. 1). Southern experiments were then carried out on complete digested clones or inserts probed with the individual rRNA species (data not shown). The location of the different hybridizing rRNA sequences within the restriction map is shown in Fig. 1.

In *T. brucei*, the small rRNAs S1–S4 and S6 are mature products from the main transcription unit [6]. In addition, sequence data from this organism indicate that S2, S4 and S6 are analogous to domain VII of other eukaryotic 28S rRNA [6]. In *C. fasciculata*, Spencer et al. have sequenced the rest of the large subunit rRNA gene [13] and have demonstrated that the coding sequence for the small rRNA molecules S1–S4 and S6 (in our nomenclature) are separated by internal DNA spacers.

In reference to the cleavage site recognition in the processing of this genetic system, Campbell et al. [23] have shown that in *T. brucei* the cleavage events that give rise to S3 and to S1 generally occur near the junction of base-paired and single stranded regions without an apparent consensus sequence.

It is likely that the above information related to *T. brucei* and *C. fasciculata* reflects structural similarities in *T. cruzi* rRNA gene.

The atypic ribosomal DNA sequence organization of *T. cruzi*, together with other unusual but shared genetic situations described in Trypanosomatids [24–26], reinforce the molecular phylogenetic observation of Sogin et al. [27] that trypanosomes represent an eukaryotic group of organisms with a deep and independent line of evolution. Sequence data from the rRNA locus in different members of the Trypanosomatidae family will be extremely valuable for the establishment of taxonomic classifications within this group, as well as for estimation of the speciation time.

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DATOS NO MOSTRADOS EN EL MANUSCRITO

La identificación de colonias portadoras de plasmidos recombinantes con DNAr se llevó a cabo mediante su hibridación _in situ_ con RNA aislado de precipitados ribosomales de 150 000 g. La figura 4 muestra el perfil electroforético (agarosa/formaldehído) del RNA usado como sonda molecular (Fig. 4A), así como el tipo de señal de hibridación observada en los autoradiogramas (Fig. 4B). Las regiones de las cajas originales (5×10^3 colonias/caja) que mostraron señal de hibridación fueron resembradas a menor densidad hasta el aislamiento de colonias independientes positivas a la sonda de RNAr.

La corroboración de que los plasmidos aislados de las colonias positivas fueran recombinantes con DNAr, fue realizada con DNA plasmidiano aislado de cultivos pequeños (1ml); La figura 5 muestra tanto el patrón electroforético de insertos clonados en los sitios _PstI_ ó _SstI_ del vehículo pUC18, como su hibridación tipo Southern con RNAr total.

Como se menciona en Materiales y Métodos, los mapas de restricción de las clonas aquí presentadas (serie pRTC) se construyeron por digestiones parciales en moléculas marcadas en un extremo. La Figura 6 ejemplifica con la clona pRTC20 el tipo de resultados obtenidos en estos experimentos.

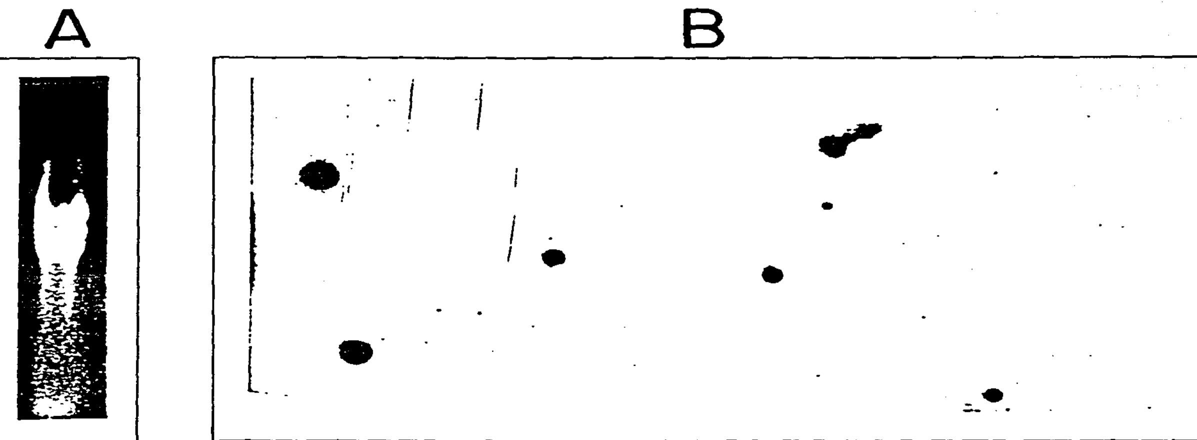


Figura 4. A: Perfil electroforético en agarosa/ formaldehído de RNAr total. Este material fue fosforilado *in vitro* mediante la encima cinasa del fago T4 y ^{32}P- γATP, para ser utilizado como sonda molecular en los experimentos de hibridación.

B: Autorradiografía de la hibridación *in situ* sobre las colonias de la biblioteca génica. La sonda radioactiva utilizada fue RNAr total fosforilado (^{32}P) *in vitro*.

Figura 5. Perfil electroforético (agarosa/bromuro de etidio) e hibridación tipo Southern de plásmidos aislados de colonias positivas a la hibridación *in situ* con RNAr total. Se muestran las construcciones ligadas en el sitio de *PstI* y las correspondientes en el sitio de *SstI* de pUC18. Como marcadores de tamaño se utilizó el vehículo linearizado (carril 6) y el DNA del fago λ digerido con *HindIII* (carril 14). Aquellos plásmidos portadores de insertos de tamaño esperado (mapa genómico previo, Fig.1) fueron seleccionados para su caracterización posterior (serie pRTC).

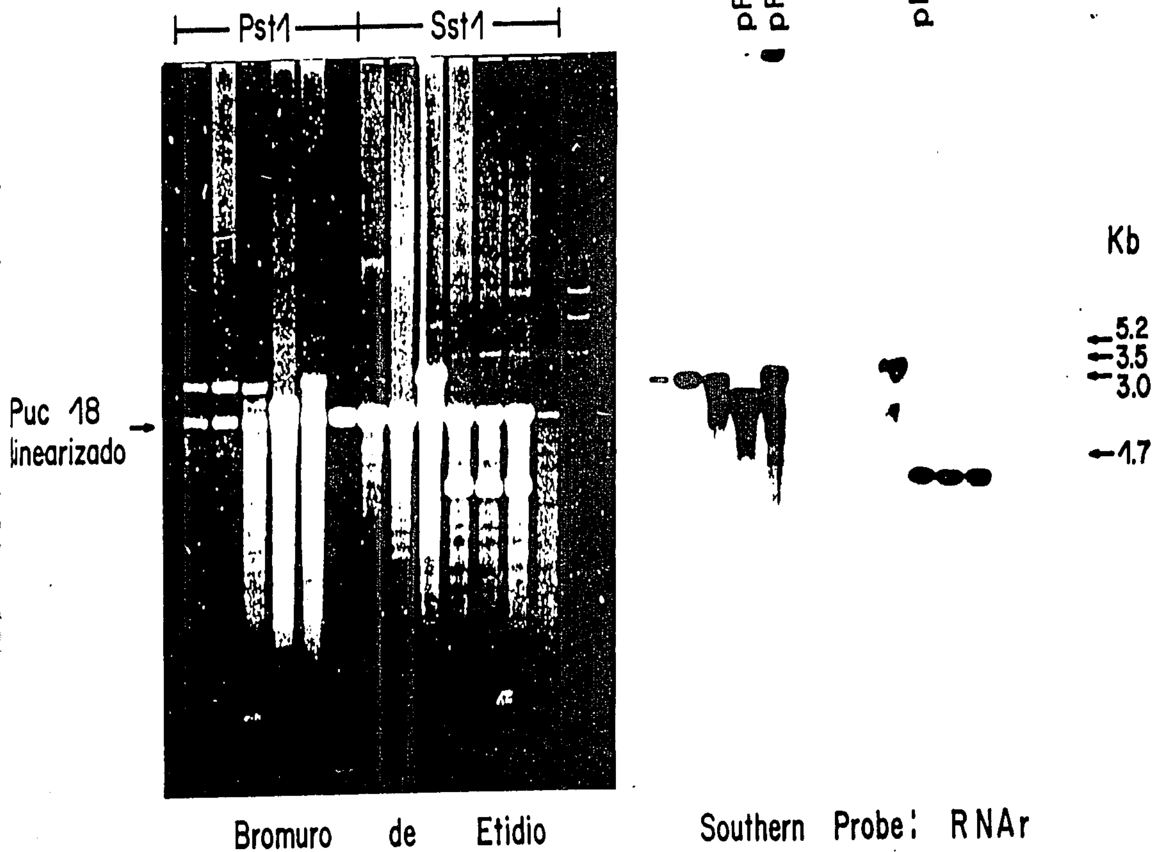
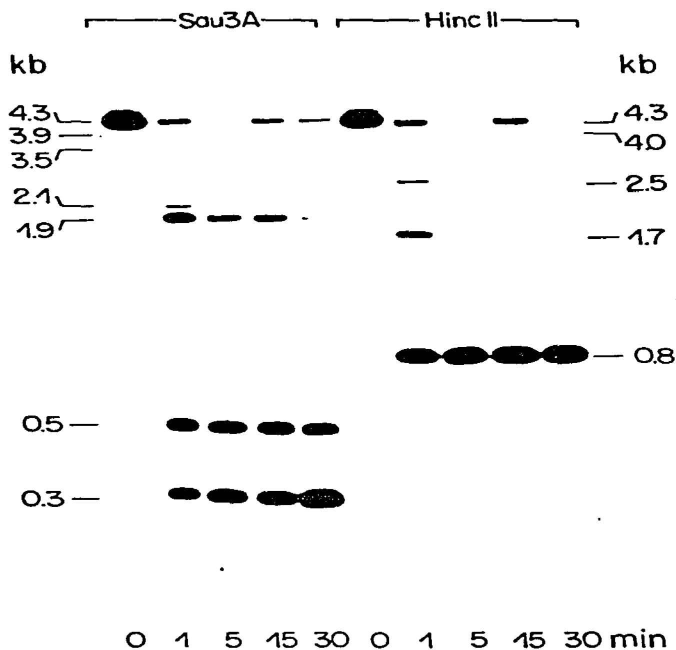


FIGURA 5

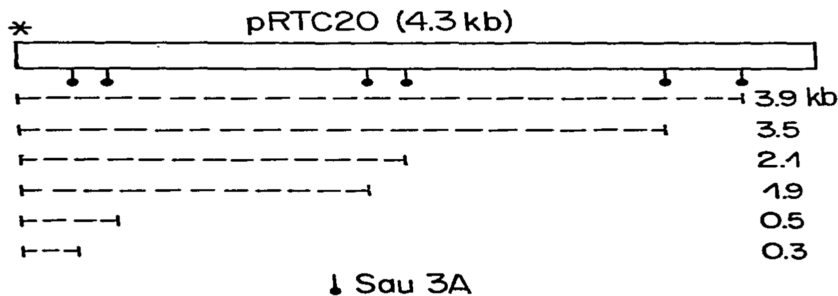
Figura 6. A: Autorradiografía de los productos de digestión parcial generados por *Sau*3A y por *Hinc*II sobre la clona pRTC20 (marcada radioactivamente en un extremo). Como se indica en la parte inferior de los carriles, las digestiones se llevaron a cabo durante 0, 1, 5, 15 y 30 minutos; y la electroforesis fue realizada en un gel de agarosa al 1%. El tamaño de los fragmentos fue calculado con los productos de digestión con *Hind*III sobre el DNA del fago λ, observados con bromuro de etidio del gel original.

B: Ubicación gráfica de los sitios de corte para *Sau*3A. La posición ordenada de los sitios resulta de la lectura progresiva en tamaño de fragmentos radioactivos, tomando como punto de referencia la posición de la marca radioactiva en el inserto de pRTC20.

A



B



Finalmente, la asignación, en los fragmentos clonados, de las diferentes regiones codificadoras de las especies maduras de RNAr, se llevó a cabo mediante experimentos tipo Southern hibridados en forma independiente con las diferentes moléculas de RNAr. La figura 7 ilustra uno de estos experimentos, realizado sobre la clona pRTC32, digerida con *HincII* o con *HinfI* e hibridada independientemente con las especies de RNAr: 24Sα, S2 y S6. La autorradiografía presentada no muestra hibridación con fragmentos menores de 300b generados por *HinfI*, sin embargo fragmentos hasta de 200b son observados tenuemente en autorradiogramas expuestos unas 10 veces mas. Este comportamiento es habitual por las limitaciones del método.

Figura 7. A: Hibridación tipo Southern sobre los productos de digestión de *Hinf*I y *Hinc*II en la clona pRTC32. El tamaño de los fragmentos de hibridación fue estimado utilizando los productos de digestión *Hind*III sobre el DNA del fago λ.

B: Ubicación de las regiones codificadoras para las especies de RNAr: 24Sβ, S2 y S6; sobre el mapa de restricción. La localización de la región que codifica para S2 fue acotada con sitios de *Sau*3A (Fig. 1). Por el tipo de experimentos realizados, la posición de las especies de RNAr sobre el mapa, es solo aproximada.

A

Hinf I Hinc II Hinf I Hinc II Hinf I Hinc II

kb

2.20

1.15

0.60

0.48

0.43

0.33

probe: S2 S6 24Sβ

B

650 ↓ 2300 b ↓ Hinc II

24Sβ S2 S6

400 ↑ 300 ↑ ↑ ↑ 250 ↑ 200 ↑ 1200 ↑ 420 ↑ Hinf I

FIGURA 7

DISCUSION

En la introducción de los artículos incluídos como "Antecedentes Particulares", se señalan las características generales de organización molecular del DNAr y los productos del procesamiento del RNAr precursor en una variedad de sistemas eucariontes.
Con intenciones de señalar los elementos "típicos" que presentan los diferentes sistemas, me permito mencionar los siguientes puntos, y comentar en cada uno de ellos la situación observada en *T. cruzi*.

1. Los genes de RNAr constituyen una familia de elementos medianamente repetidos (50- 500 copias por genoma haploide) |20|. *T. cruzi* contiene alrededor de 110 genes por núcleo |24|.
2. Las unidades de transcripción de RNAr se encuentran agrupadas. Desconocemos la ubicación del cistrón de RNAr en *T. cruzi*, pero el hecho de haber encontrado un patrón relativamente sencillo de bandas genómicas de hibridación, sugiere que las unidades de transcripción se encuentran en un contexto homogéneo de secuencias. Cabe mencionar que en las especies africanas (*T. brucei*) , los genes para RNAr se encontraron agrupados en 3 o 4 *locus* de cromosomas diferentes; a su vez separados de los genes codificadores de RNA 5S |25|.
3. Las unidades de transcripción de RNAr se encuentran separadas por regiones de DNA no transcritas. Tal es el caso en *T. cruzi*, y los espaciadores no transcritos resultan ser de por lo menos 20kb, y heterogéneos en secuencia y/o en longitud (Fig. 3)

4. Las unidades de transcripción contienen espaciadores transcritos en el RNAr precursor, y al ser eliminados durante el procesamiento del mismo se generan las moléculas maduras de RNAr: 18S, 5.8S y 28S (como valores nominales). La organización intracistrónica del DNAr en T. cruzi es el objeto fundamental de esta Tésis, por lo que me permito desglozar las consideraciones correspondientes en los párrafos siguientes.

Los datos de organización presentados, indican que las moléculas nóveles de RNAr de T. cruzi, se encuentran codificadas en un arreglo atípico al resto de eucariotes (Fig. 8) y virtualmente idéntico al reportado recientemente en los Tripanosomatídeos T. brucei |26| y Crithidia fasciculata |27| . Por datos de secuencia nucleotídica en estos dos últimos organismos se demostró la correspondencia de las moléculas pequeñas de RNAr (excepto S5) con regiones del RNA de la subunidad mayor ribosomal. La aportación del presente trabajo reafirma el concepto de un modelo génico de organización compleja y particular al grupo filogenético de Tripanosomatídeos. Dicho modelo se caracteriza fundamentalmente por la presencia de DNA espaciador en sitios no descritos anteriormente que estructura un gene discontínuo. Esta organización desusual tiene tanto implicaciones estructurales como evolutivas.

Implicaciones estructurales

La correspondencia, por un lado, de la especie de RNA 5.8S de ribosomas citoplásmicos, y por otro, de la molécula 4.5S de ribosomas de cloroplastos, con las regiones 5' y 3' del RNAr 23S de E. coli respectivamente |28|; indica que no se requiere colinearidad estricta y covalente a lo largo de la molécula de RNAr para el funcionamiento de la subunidad ribosomal mayor.

En algunos organismos protozoarios |29- 32| , y algunos metazoarios del tipo protostomados |33- 34| existe la discontinuidad interna y aproximadamente central en la mencionada especie de RNAr mayor, que separa a la molécula en dos entidades (α y β). Aún mas, en Drosophila se presenta un procesamiento extraordinario de la molécula 5.8S que da orígen a una molécula 2S adicional |35|. En todos estos casos, sin embargo, el complejo molecular de RNAr (aunque discontinuo) permanece unido por enlaces de puente de hidrógeno aún en ausencia de proteínas.

En T. cruzi, y por trabajo realizado en nuestro laboratorio sabemos que las interacciones intermoleculares de RNAr estabilizadas por puents de hidrógeno se presentan entre las dos moléculas mayores (α y β), y entre S3 y la molécula α. El resto de las moléculas pequeñas se liberan de la subunidad ribosomal mayor en ausencia de proteínas (Antecedentes Particulares, |36|). La asociación de la mayoría de las moleculas de RNAr pequeñas

a los ribosomas, depende entonces de la estabilización dada por proteínas.

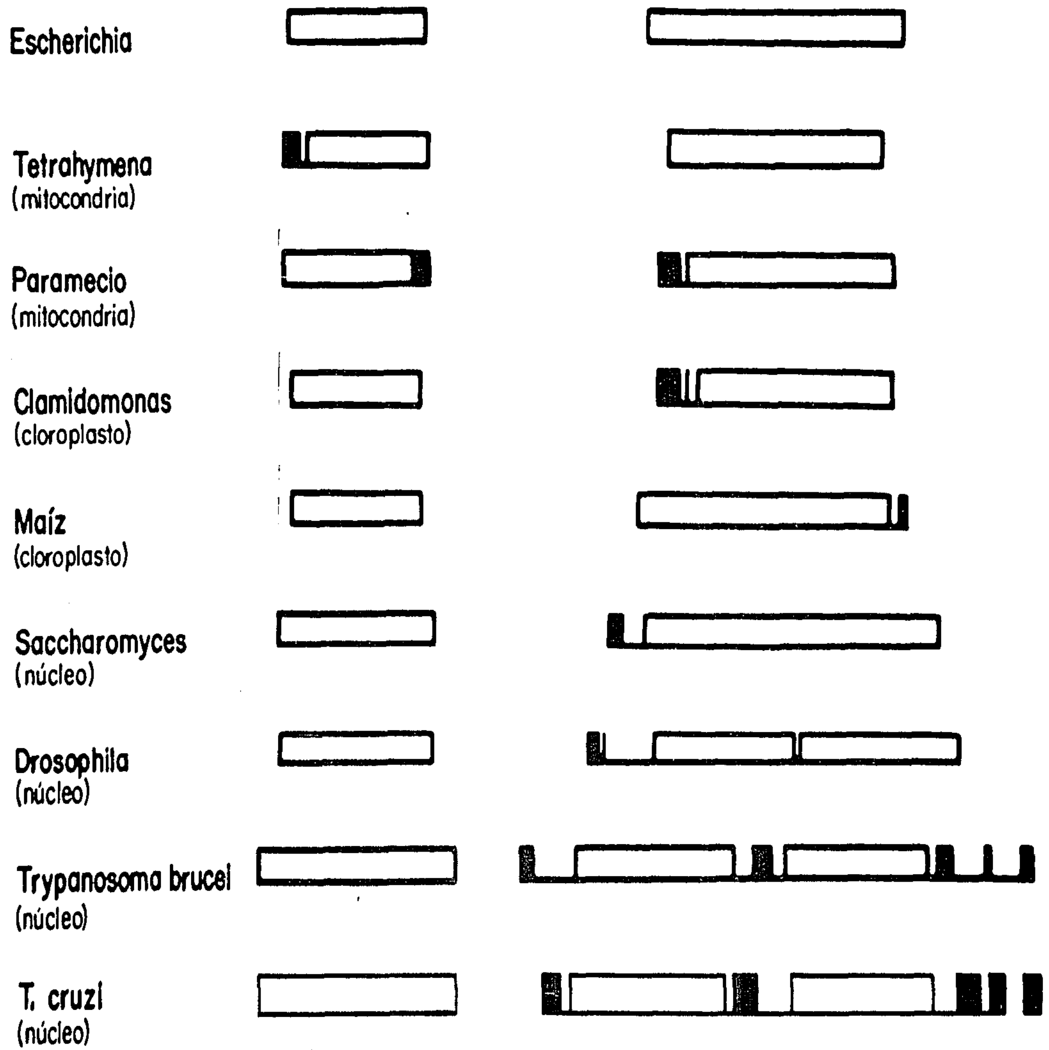
El tener dominios funcionales segmentados de la especie de RNAr tipo 28S, asociados por proteínas a la subunidad ribosomal mayor no tiene precedentes en ribosomas citoplásmicos de eucariontes. Esta situación, refleja posiblemente una estructura RNA-proteína diferente en estos organismos, que consideramos de importancia estudiar.

Consideraciones evolutivas

Para ilustrar el significado de la organización en el DNAr y sus productos de procesamiento en Tripanosomas, en comparación a otro tipo de genomas, me permito utilizar la revisión ilustrada en |26|, donde incluyo el arreglo construído en el presente trabajo (Fig. 8). Tanto el procesamiento de S1 dado en el espaciador trasncrito entre α y β, como el procesamiento de S2, S6, y S4 en el extremo 3' del cistrón, son ejemplos no usuales de organización en genomas eucariontes. Por otro lado, procesamientos desusuales se encuentran en el RNAr de mitocondrias y plástidos de protozoarios y de plantas. Los cloroplastos de estas últimas, presentan incluso un producto de procesamiento hacia el extremo 3'. Sin embargo, ningún organismo descrito a la fecha, presenta una

Figura 8. Esquema tomado de White, T.C. y cols. |26| donde se muestran diferentes sistemas biológicos de procesamiento de RNAr. Tanto las especies de RNA presentes en la subunidad mayor, como aquellas presentes en la subunidad menor ribosomal son mostradas en las diferentes columnas. Las especies pequeñas de RNA se esquematizan como cajas negras y aquellas de mayor tamaño como cajas vacías. En la parte inferior de la figura se insertó el arreglo génico encontrado para *T. cruzi* por el presente trabajo. Las referencias originales de los datos aquí presentados se encuentran en la fuente ya mencionada |26|.

ESTA TESIS NO DEBE SALIR DE LA BIBLIOTECA


RNAr: SUBUNIDAD MENOR
SUBUNIDAD MAYOR
Escherichia
Tetrahymena
(mitocondria)
Paramecio
(mitocondria)
Clamidomonas
(cloroplasto)
Maíz
(cloroplasto)
Saccharomyces
(núcleo)
Drosophila
(núcleo)
Trypanosoma brucei
(núcleo)
T. cruzi
(núcleo)

complejidad comparable a la descrita en los tres miembros estudiados de la familia de Tripanosomatídeos.

La presencia sin precedente de 8 moléculas de RNAr citoplásmico, organizado en forma similar en dos especies de tripanosomas y en un organismo del género Crithidia, sugiere que la familia de Tripanosomatídeos evolucionó de forma independiente (antigua) al resto de eucariontes. Para aumentar la complejidad del cuadro general, la organización génica de las moléculas de RNAr (9S y 12S) mitocondrial en los maxicírculos de estos cinetoplástidos violan la regla general de polaridad 5'-3' |37|; pues a la inversa de cualquier otro genoma a la fecha descrito (procarionte, mitocondrial, plástido o nuclear), las especies de RNAr mencionadas se encuentran codificadas en la polaridad opuesta:

5' ——12S—— ——9S—— 3'

Como ya fue mencionado en la Introducción, la secuencia de RNAr ofrece información con la que se pueden construír árboles filogenéticos |23|. Sogin y cols. |38| han construído árboles filogenéticos comparando la secuencia del RNAr de la subunidad menor (RNAr tipo 18S); y entre los organismos analizados incluyeron a T. brucei |38| y a C. fasciculata |39| . Las conclusiones que sus estudios arrojaron fueron que los dos Tripanosomatídeos analizados, evolucionaron relacionados entre sí, y de manera independiente no sólo al resto de eucariontes, sino también a otros

protozoarios considerados "cercanos" bajo otros criterios |41|. El ũnco protista (a la fecha conocido) relacionado con la línea evolutiva de los Tripanosomatídeos, está representado por <u>Euglena</u> |38- 40|. Aparentemente (datos no publicados pero mencionados), organismos Euglénidos presentan también productos adicionales de procesamiento en regiones similares a las de Tripanosomatídeos |27|.

En cuanto al orígen de estos productos nuevos de procesamiento y a su relación con el RNAr tipo 23- 28S, existen dos posibilidades:

(a) El genoma nuclear progenitor tuvo una secuencia codificadora contínua para la molécula de RNAr de la subunidad mayor, pero en la dirección evolutiva de los Tripanosomatídeos, y por eventos de inserción de DNA espaciador, se separaron las regiones codificadoras de las distintas moléculas maduras de RNAr.

(b) El gene para la molécula mayor de RNAr en el genoma eucarionte primigenio fue ciertamente discontinuo, y los espaciadores fueroneliminados diferencialmente en las líneas evolutivas posteriores. Bajo ésta hipótesis, los Tripanosomatídeos, al haber evolucionado en una línea antigua y diferente al resto de eucariontes, pudieron haber conservado los espaciadores ahora descritos.

El hecho de que el gene de la subunidad grande ribosomal de los microscorídeos, que a su vez representan a la fecha la línea evolutiva independiente mas antigua de eucariontes resulte continuo |40|, aún sin procesamiento de la molécula 5.8S, haría pensar que la primera posibilidad (progenitor contínuo) como factible.

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