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COMPLEXITY AND CONTENT OF THE DNA AND RNA IN *TRYPANOSOMA CRUZI*

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The content and sequence complexity of the nuclear DNA and messenger RNA for epimastigotes of *Trypanosoma cruzi* were determined. From analysis of nuclear DNA reassociation studies and microspectrofluorometric measurements of laser induced fluorescence of cellular DNA, *T. cruzi* is found to be a diploid organism with a nuclear DNA content of 2.5×10^8 nucleotide pairs (2.8×10^{-13} g) and a kinetoplast DNA content of 4.9×10^7 nucleotide pairs (5.4×10^{-14} g). Reassociation kinetics of nuclear DNA of average length 0.4 kb reveals three kinetic components: a moderately repetitive component with a reiteration frequency of 5.1×10^3 present in 9% of the fragments, a lowly repetitive component with a reiteration frequency of 32 present in 51% of the fragments, and a single-copy component present in 23% of the fragments.

By saturation hybridization of total polysomal RNA to ^{3}H-labeled single-copy DNA, it was determined that 68% of the single-copy DNA was represented in the epimastigote polysomal RNA. This corresponds to ca. 12 000 different mRNA species. Of these, ca. 9000 are present as poly(A)$^+$-RNA, while the remaining 3000 appear not to be polyadenylated. Kinetic analysis of the poly(A)$^+$-RNA population indicates it is composed of at least three classes of RNAs of different abundancy levels: two sequences which occur ca. 3000 times per cell, ca. 750 sequences which occur about 20 times per cell, and ca. 15 500 sequences which occur 1–2 times per cell.

Key words: *Trypanosoma cruzi*, RNA, DNA, Reassociation kinetics, Sequence complexity.

INTRODUCTION

Trypanosoma cruzi is distinguished among parasitic flagellates as the causative agent of Chagas' disease. Information is now available concerning its energy and nucleic acid metabolism [1], as well as the mode of replication [2] and restriction endonuclease analysis [3] of its kinetoplast DNA. However, an area about which information is particularly limited is that of the structural organization and expression of the genomic DNA.

Abbreviations: C_0t, R_0t, initial DNA or RNA concentration in mol nucleotides × time (s). kb, kilobases, 1000 nucleotides or nucleotide pairs for single- and double-stranded DNA, respectively; NT, nucleotides; NTP, nucleotide pairs; RMS, root mean square; poly(A)$^+$-RNA, polyadenylated RNA; poly(A)$^-$-RNA, non-polyadenylated RNA; FCS, fetal calf serum.

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This is a subject of intense interest since the developmental cycle of *T. cruzi* must eventually be directed by the modulation and expression of the information in the nuclear genome. We have, therefore, conducted studies directed at measuring the size, ploidy, and extent of expression of the genomic DNA of *T. cruzi*.

MATERIALS AND METHODS

Culture conditions. Trypanosoma cruzi, strain Y, was grown in 1 l of Warren's medium (Brain Heart Infusion/10% heat-inactivated fetal calf serum (FCS)/3 mg/l hemin), without shaking in Fernbach flasks at 28°C to a cell concentration of ca. 1×10^7 cells/ml. Log-phase division time under these culture conditions was about 20 h. 12 h before cell harvest 100 ml fresh Warren's medium with 20% FCS was added to maintain logarithmic growth. The cells were harvested at a concentration of ca. 2×10^7 cells/ml by centrifugation at $3000 \times g$ for 10 min at 4°C. The cells were washed twice with Hanks' Buffered Saline with 1% bovine serum albumin and used immediately for preparation of either DNA or polysomal RNA. Epimastigote numbers were determined by Neubauer hemacytometer counts using phase microscopy.

Preparation of DNA. T. cruzi DNA was extracted from whole cells by the procedure previously described for the extraction of DNA from *Drosophila melanogaster* nuclei [4]. Nuclear DNA was purified from preparations of total cellular DNA (nuclear DNA + kinetoplast DNA) by CsCl density-gradient centrifugation [5]. ^{32}P- or ^{3}H-labeled *T. cruzi* DNA was obtained by culturing cells to 2×10^7 cells/ml in medium containing 5 mCi/l [^{32}P]thymidine (New England Nuclear (NEN), Boston, MA), or 1 mCi/l [^{3}H]thymidine (NEN). The specific activity of a typical preparation of ^{32}P- or ^{3}H-labeled DNA was 1.1×10^2 cpm/μg or 4.1×10^5 cpm/μg, respectively.

Preparation of ^{3}H-labeled single-copy DNA. Unlabeled single-copy DNA was prepared by several cycles of reassociation and hydroxyapatite fractionation [6]. Briefly, nuclear DNA fragments of a starting average single-strand length of 950 nucleotides (NT) were reassociated to C_0t 100 (initial concentration of DNA in moles nucleotides × time(s)) and the duplex fraction removed. The single-stranded fragments were again reassociated to C_0t 100 and the duplex fraction removed. The remaining single-stranded DNA was reassociated to completion (C_0t 1000 for *T. cruzi* DNA) and labeled in vitro with [^{3}H]-TTP (NEN; 62 Ci/mmol) using *Escherichia coli* DNA polymerase I (Boehringer-Mannheim Grade I) as previously described [7]. Foldback regions were removed by hydroxyapatite chromatography as described [6]. DNA prepared in this manner had a final average single-strand length of 210 NT and a specific activity of 2×10^6 cpm/μg. The reactivity of this DNA was approximately 88% when reassociated in trace quantities with excess amounts of total *T. cruzi* nuclear DNA.

^{3}H-labeled single-copy DNA was also prepared from in vivo labeled nuclear DNA by the several cycles of reassociation and hydroxyapatite fractionation described above.

Denaturation and renaturation of DNA fragments. The experimental procedures used for shearing of DNA, denaturation, reassociation, hydroxyapatite chromatography and C_0t curves were as previously described [4, 6].

Microfluorometry of DNA. The use of laser induced fluorescence for quantitation of nuclear DNA content is described in detail elsewhere [8, 9]. In these studies, the DNA content of both the nucleus and kinetoplast of logarithmically dividing cells was measured. One hundred measurements of each were taken and the DNA content was calculated, using as an external calibration the DNA content of *Escherichia coli* strain C600. The DNA content of the nucleus and the kinetoplast of *T. cruzi* were determined to be $2.5 \pm 0.2 \times 10^8$ nucleotide pairs (NTP) and $4.9 \pm 0.3 \times 10^7$ NTP, respectively. The total cellular (nucleus + kinetoplast) DNA of *T. cruzi* is, then, 3.0×10^8 NTP with the kinetoplast DNA comprising 16% of the total. It should be noted that this particular technique has been used previously [8] to delineate accurately diploid from tetraploid populations. The later distinction has been confirmed by karyotype analysis of mitotic spreads.

Isolation of polysomes and polysomal RNA. Polyribosomes were isolated from epimastigotes in exponential growth phase by a modification [6] of the procedure of Jackson and Larkins [10]. Following sedimentation in exponential sucrose velocity gradients (Fig. 1A), polysomes sedimenting at > 100 S were collected and concentrated by centrifugation. Polysomal RNA was extracted with phenol/chloroform (1 : 1) and further purified by centrifugation through 6 M CsCl followed by chromatography over a column containing Chelex (Biorad 200–400 mesh) and Sephadex G-100. RNA excluded from this column was ethanol-precipitated and stored at -70°C.

Preparation and sizing of poly(A)$^+$ RNA. Poly (A)$^+$-RNA was isolated from total polysomal by oligo(dT)-cellulose chromatography [11]. Poly(A)$^+$-RNA purified by three cycles of oligo(dT)-cellulose chromatography and sized by gel electrophoresis in the presence of the denaturing agent CH_3HgOH [12] exhibits a mass average length of 1350 NT (Fig. 1B), and represents $3.7 \pm 0.2\%$ of the total mass of the polysomal RNA.

DNA–RNA hybridizations with gap-translated ^{3}H-labeled single-copy DNA. The hybridization of total polysomal RNA with gap-translated ^{3}H-labeled single-copy DNA was measured by reacting a 2000–10 000-fold mass excess (200–800 μg) of purified polysomal RNA with trace quantities (ca. 0.08–0.16 μg) of ^{3}H-labeled single-copy DNA. Reactions were conducted in 0.75 M NaCl/2.5 mM PIPES (Sigma), pH 6.8/0.2% SDS, at 65°C in sealed capillary pipettes [6].

The hybridization of poly(A)$^+$-polysomal RNA to ^{3}H-labeled single-copy DNA was measured by hybridization of a 1000-fold mass excess (23 μg) of purified poly(A)$^+$-polysomal RNA with trace amounts (0.023 μg) of ^{3}H-labeled single-copy DNA. Reaction conditions were identical to those described for the hybridization of total polysomal

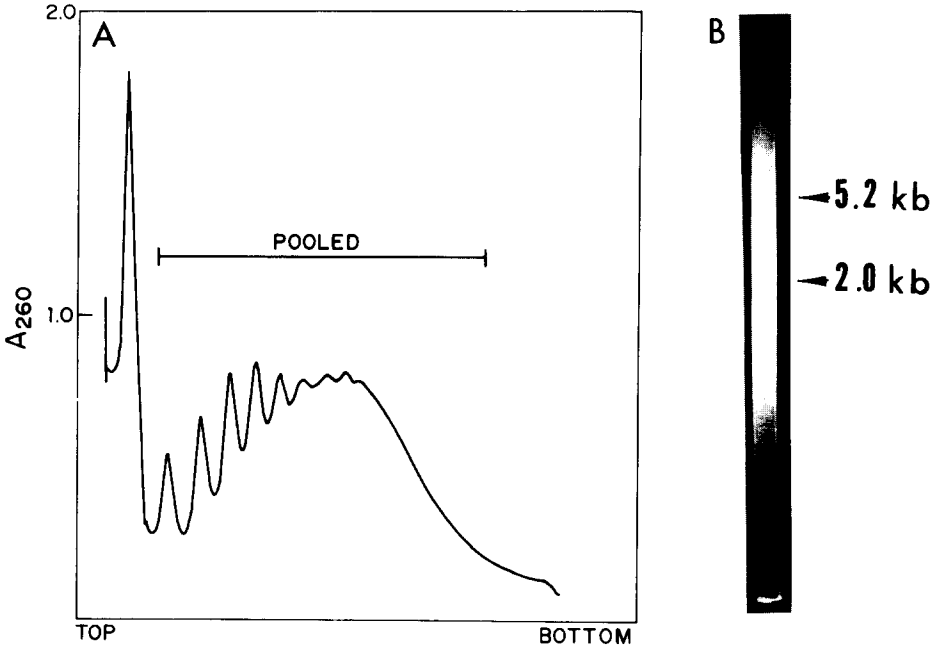

Fig. 1. (A) Preparative polyribosome sedimentation profile. The post-mitochondrial supernatant from epimastigotes was layered onto exponential sucrose gradients (0.5–1.5 M sucrose) and sedimented at 23 000 rev./min for 3.5 h in an SW-27 rotor. Gradients were fractionated through a flow cell, and the fractions indicated by the bar were pooled and the RNA extracted. (B) Electrophoretic profile of poly(A)$^+$-RNA on a CH_3HgOH denaturing gel.

RNA. Heterologous yeast tRNA (776 μg) was added to each reaction to make total RNA concentrations similar to those in total polysomal RNA hybridizations. R_0t (initial RNA concentration in moles nucleotides X time(s)) values were calculated based upon the concentration of *T. cruzi* RNA.

In order to assess, and correct for, the contribution of DNA–DNA self-reassociation to the total amount of hybridization in the RNA–DNA reactions, control samples were prepared as described above, substituting appropriate amounts of yeast tRNA for total polysomal or poly(A)$^+$-RNAs.

Synthesis of [^{3}H]cDNA to poly(A)$^+$-mRNA. Synthesis of [^{3}H]cDNA was carried out in a 10 μl reaction volume as follows: 4 μg *T. cruzi* poly(A)$^+$-mRNA was co-precipitated with 200 μg oligo(dT) (Cal Biochem) by addition of 2 vols. of 95% ethanol. The precipitate was dried with a gentle stream of N_2 and resuspended in 10 μl of 2X cDNA buffer (40 mM $MgCl_2$/200 mM Tris, pH 8.3). In a separate reaction vessel, 1 mM each of dGTP, dATP, dCTP and [^{3}H]dTTP were evaporated to dryness. 5 μl of the resuspended poly(A)$^+$-mRNA/oligo(dT) mixture (2 and 100 μg, respectively), 1 μl 300 mM dithiothreitol, 1 μl freshly prepared 10 mg/ml actinomycin D, 1.6 μl chelexed H_2O and 1.4 μl

reverse transcriptase (12.5 U) (courtesy of Dr. J. Beard, Life Sciences, Inc., FL) were then added to the dried nucleotides. Up to this point all operations were carried out at 4°C. The mixture was then incubated at 46°C for 1 h and the reaction terminated by the addition of 20 μg *E. coli* double-stranded DNA and 300 μl 0.1 N NaOH, 0.2% SDS and incubated for 10 min at 95°C. The mixture was then neutralized with 100 μl 2 M Tris-HCl, pH 4, and extracted with 400 μl $CHCl_3$/octanol (8 : 1). The aqueous phase was chromatographed on Sephadex G-100 and the excluded fractions were pooled and ethanol-precipitated. The precipitate was collected by centrifugation, resuspended in 500 μl of 0.2 M sodium acetate, layered on a 5–20% sucrose gradient, and centrifuged at 30 000 rev./min for 15–1/2 h, at 4°C in a Beckman SW-41 rotor. The gradient was fractionated and those peak fractions containing the [^{3}H]cDNA were pooled and ethanol precipitated with carrier tRNA at a final concentration of 36 μg/ml. The mass average length of the [^{3}H]cDNA molecules was ca. 550 nucleotides as determined by gel electrophoresis in the presence of CH_3HgOH [12], and had ca. 0.7% foldback as determined by resistance to S_1 nuclease, and a specific activity of 4×10^7 cpm/μg.

Polysomal poly(A)$^+$–[^{3}H]cDNA hybridization. The hybridization of polysomal poly-(A)$^+$-RNA with [^{3}H]cDNA was measured by reacting a 1400–6500-fold mass excess (0.44–2.0 μg) of purified polysomal poly(A)$^+$-RNA with trace quantities of [^{3}H]cDNA (3.1×10^{-4} μg). Reaction conditions were identical to those described above for the hybridization of poly(A)$^+$-RNA and ^{3}H-single copy DNA.

Assay of hybridization. Hybridized samples were resuspended in 250 μl of 30 mM sodium acetate/0.15 M NaCl/0.1 mM $ZnSO_4$/5% glycerol, pH 4.3, containing 15 μg native and 10 μg denatured *E. coli* DNA. An aliquot (25 μl) was removed to measure total radioactivity per sample. One thousand units of S_1 nuclease (Boehringer Mannheim) were added samples were incubated for 30 min at 37°C. S_1 nuclease resistance was measured by fractionation of the sample by Sephadex G-100 chromatography. The ratio of counts in the excluded volume to the total counts of the included and excluded fractions yielded the S_1 nuclease resistance in each sample. Recovery of the input [^{3}H]cDNA was determined to be $> 95\%$ as measured by summation of the radioactivity in the included and excluded volumes.

RESULTS

Reassociation kinetics of Trypanosoma cruzi *nuclear DNA.* Fig. 2 shows the reassociation kinetics of nuclear DNA fragments of average length 0.4 kilobases (kb), using standard hydroxyapatite chromatography conditions. Reassociation of the fragments occurs over at least five decades of C_0t and the curve that best fits the data points as determined by least squares analysis has three kinetic components. As shown in Table I and Fig. 2, analysis of the reassociation profile of fragments of average length 0.4 kb identified a moderate repetitive component with a reiteration frequency of 5.1×10^3 present in 9%

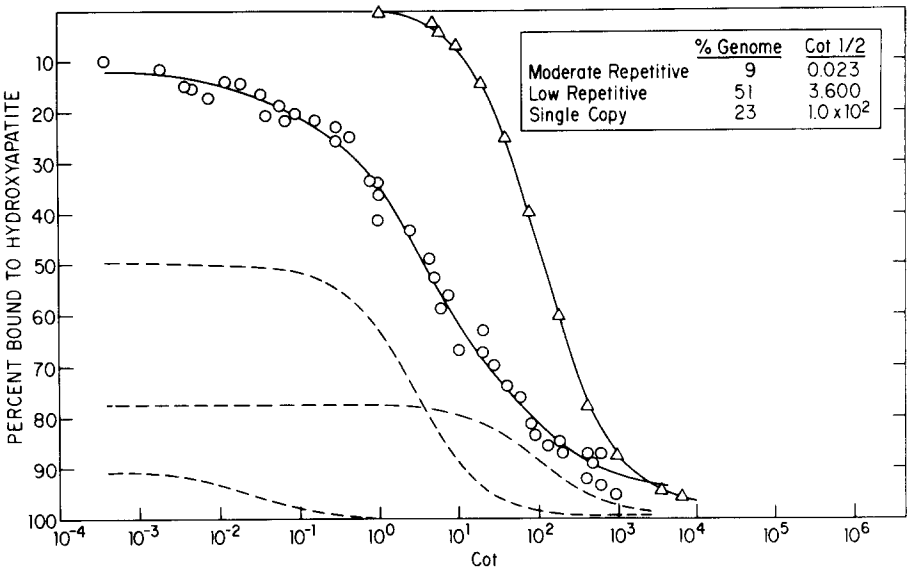

Fig. 2. Hydroxyapatite reassociation kinetics of *T. cruzi* DNA. *T. cruzi* DNA of fragment length 0.4 kb was reassociated as described in Materials and Methods. The fraction of fragments containing duplex regions was measured at the indicated equivalent C_0t values by hydroxyapatite chromatography. The curve for the 0.4 kb fragments (○) represents a computer analysis for three second order components. The RMS value of this solution is 0.031. To more accurately determine the rate constant for the single copy component, a fraction of ^{3}H-labeled 0.4 kb tracer DNA was mixed with a 400-fold excess of total 0.4 kb unlabeled driver DNA. The curve (△) represents a least-squares analysis for one second-order component. The dashed curves represent the components of the computer solution which, when summed, give the total curve. The RMS value of this solution is 0.003 (see Table I).

of the fragments, a low repetitive component with a reiteration frequency of 32 present in 51% of the fragments, and a single-copy component present without repetitive DNA elements in 23% of the genome. 12% of the DNA fragments bind to hydroxyapatite at a C_0t less than 3×10^{-4}, and, thus, represent foldback or zero-time binding DNA. Also, 5–7% of the DNA sequences failed to form stable duplex structures at the highest values of C_0t attained, possibly as the result of limited fragment length.

In order to accurately determine the reassociation rate constant for the putative single copy sequences, trace amounts of ^{3}H-labeled DNA, enriched for single copy sequences, were prepared. Total ^{3}H-labeled nuclear DNA fragments were reassociated to C_0t 100 and the reassociated fragments were removed by binding to hydroxyapatite. The unbound fragments were again reassociated to C_0t 100 and the duplex structures removed by hydroxyapatite chromatography. The unbound fragments were once again reassociated to C_0t 1000 and the duplex [^{3}H]DNA collected. The final fraction that bound to hydroxyapatite (single-copy DNA) was denatured and then reassociated with excess total nuclear *T. cruzi* DNA. As shown in Fig. 2, the tracer DNA is highly enriched for the putative single-copy sequences.

TABLE I

Kinetic analysis of *T. cruzi* DNA.

Component	Fraction[a] of fragments	K_{pure}[b] ($M^{-1} \cdot s^{-1}$)	K_{whole}[c] ($M^{-1} \cdot s^{-1}$)	$C_0t_{1/2}$ whole[c]	No. of copies per genome	Kinetic complexity[d]
Foldback	0.12					
Moderately repetitive	0.09	4.8×10^2	43	0.023	5.1×10^3	2.1×10^3
Lowly repetitive	0.51	0.55	0.28	3.6	32	1.9×10^6
Single copy	0.23	4.3×10^{-2}	1.0×10^{-2}	1.0×10^2	1	2.4×10^7
Single copy tracer	0.95		9×10^{-3}	1.1×10^2	1[e]	1.2×10^8

[a] Computed from the reassociation analysis of 0.4 kb DNA fragments and the renaturation kinetics of ^{3}H-labeled single-copy tracer.

[b] K_{pure} ($M^{-1} \cdot s^{-1}$) is the parameter which describes the reassociation of a homogenous kinetic component of which 100% of the reassociated DNA fragments bind to hydroxyapatite. The K_{pure} value is computed from the empirical K value obtained from reassociation of DNA fractions enriched in the kinetic components. The relationship between the empirical k and the K_{pure} is $K_{pure} = K/Q$, where Q is the amount of the fraction comprising the kinetic component.

[c] K_{whole} and $C_0t_{1/2\ whole}$ are kinetic parameters which describe the reassociation of a kinetic component in the presence of unfractionated DNA. The relationship between K_{whole} and $C_0t_{1/2\ whole}$ is $C_0t_{1/2\ whole} = 1/K_{whole}$.

[d] The kinetic complexity is expressed in nucleotide pairs. These values were computed relative to the complexity of *E. coli* DNA (4.2×10^6 NTP) [30] and the rate constant of 0.4 kb fragments of *E. coli* DNA ($0.25\ M^{-1} \cdot s^{-1}$).

[e] Assumed value of 1 for the single-copy component.

It is possible to determine whether this class of slowly reassociating sequences is single copy by comparing the genome size determined by the reassociation rate constant of the putative single copy DNA with that obtained by direct chemical analysis [13, 14]. Under the reassociation conditions used in these experiments, the rate constant for the presumed single copy sequences in *T. cruzi* nuclear DNA is $9 \times 10^{-3}\ M^{-1} \cdot s^{-1}$. Therefore, based upon this measurement, the haploid genome contains 1.2×10^8 NTP of DNA.

A chemical determination of the amount of DNA in logarithmically dividing *T. cruzi* was performed by quantitation of the laser beam-induced fluorescence of DNA in Feulgen-stained nuclei. The results indicate an average value of 2.5×10^8 NTP of DNA per nucleus. This value is in reasonable agreement with the 2.4×10^8 NTP/nucleus (1.2×10^8 NTP/haploid genome $\times$ 2) expected for a diploid genome based on our kinetic measurements. We conclude, therefore, that the slowly reassociating sequences are single-copy DNA and that *T. cruzi* is most likely a diploid organism.

Sequence complexity of poly(A)$^+$- and total polysomal-mRNA. In order to determine what fraction of the sequence complexity of the single-copy DNA is being expressed in the epimastigote stage, the sequence complexity of the polysomal mRNA was measured.

In view of the fact that the total sequence complexity of the mRNA in several organisms or organs [6, 15, 16] has been shown to exist in discrete subpopulations of non-adenylated and adenylated species, it was desirable to ascertain the relative contribution of both to the total complexity. Therefore, either total polysomal mRNA or poly(A)$^+$-polysomal mRNA was hybridized to ^{3}H-labeled single-copy DNA (Fig. 3) and the level of hybridization observed at saturation was used to estimate the sequence complexity of the mRNA.

Sequence complexity of polysomal RNA. In order to measure the sequence complexity of total polysomal mRNA (i.e., nonadenylated + adenylated mRNA), polysomal RNA was hybridized in excess to trace quantities of ^{3}H-labeled single-copy DNA (Fig. 3A). At appropriate R_0t values, samples were removed and assayed for hybrid content by resistance to S_1 nuclease digestion. As summarized in Table II, the fraction of a single-copy DNA which hybridizes with the mRNA at saturation provides a direct estimate of the polysomal mRNA complexity.

At saturation ($R_0t > 2000$), 30% of the ^{3}H-labeled single-copy DNA hybridized with polysomal mRNA. After correction for tracer DNA reactability (88%) and asymmetric transcription, 68% of the single-copy DNA in the *T. cruzi* genome is represented in epimastigote polysomal mRNA. This corresponds to 1.6×10^7 NT of mRNA coding sequences, or approximately 11 850 different mRNA sequences of average size 1350 NT.

Sequence complexity of poly(A)$^+$-mRNA. The question of whether the sequence complexity of polysomal mRNA is entirely represented by the polyadenylated mRNA population can be answered by comparing the saturation hybridization value of ^{3}H-labeled single-copy DNA with polysomal mRNA to that of ^{3}H-labeled single-copy DNA and

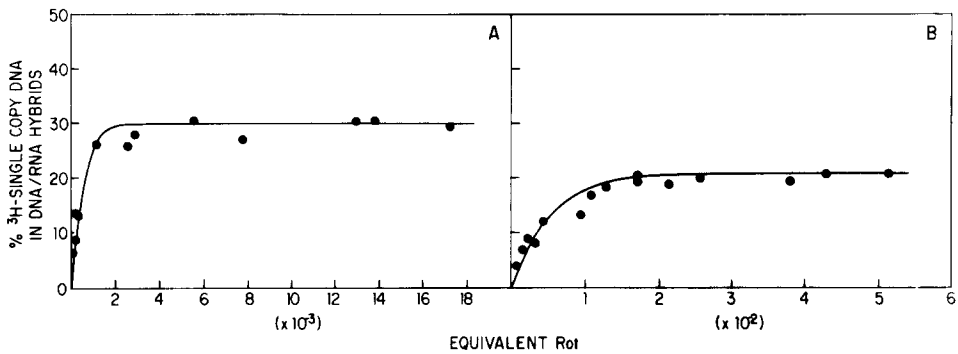

Fig. 3. Saturation hybridization of ^{3}H-labeled single-copy DNA to *T. cruzi* RNA. Trace quantities of ^{3}H-labeled single-copy DNA were hybridized with a 2000–10 000-fold mass excess of total polysomal RNA (A), or with a 1000-fold mass excess of polysomal poly(A)$^+$-mRNA (B). Hybridization was monitored by resistance to S_1 nuclease digestion. Data points have been corrected for the contribution of ^{3}H-labeled single-copy DNA self-reassociation to the total hybridization.

poly(A)$^+$-mRNA. The results of the hybridization of polysomal poly(A)$^+$-mRNA to ^{3}H-labeled single-copy DNA are presented in Fig. 3B and summarized in Table II.

At saturation ($R_0t > 180$) 21% of the ^{3}H-labeled single-copy DNA hybridized to the poly(A)$^+$-mRNA. After correction for asymmetric transcription and tracer reactability (86%), we calculate that 49% of the coding capacity of the ^{3}H-labeled single-copy DNA sequences is represented in the poly(A)$^+$-mRNA population. This value corresponds to a sequence complexity of 1.2×10^7 NT, or approximately 8900 diverse mRNA sequences of average size 1350 NT. This represents a decrease of about 3000 mRNA species from that observed in the total polysomal mRNA, and clearly indicates that approximately 25% of the total mRNA population is contributed by nonadenylated mRNA.

Polysomal poly(A)$^+$-mRNA–[^{3}H]cDNA hybridization. The experiments described above represent the most accurate quantitative approach for the measurements of the sequence complexity of total polysomal mRNA [6, 17–19]. However, this approach cannot accurately assess the number and distribution of mRNA species which may comprise a significant proportion of the mRNA mass, but a low fraction of the total mRNA sequence complexity. Also, since the measurement of the complexity of the mRNA population is based upon hybridization of mRNA and ^{3}H-labeled single-copy DNA, the contribution of mRNA species coded by repetitive DNA is refractory to analysis.

In view of these limitations, the complexity and distribution of polysomal poly(A)$^+$-mRNA were estimated by hybridizing it with [^{3}H]cDNA. The poly(A)$^+$-mRNA was used as a template for the preparation of [^{3}H]cDNA with avian myeloblastosis virus reverse transcriptase in the presence of oligo(dT) as a primer. Because the mRNA is the template for [^{3}H]cDNA synthesis, the percentage by mass of any individual mRNA species is proportionately represented in the resultant [^{3}H]cDNA population. Thus, mRNA populations of high abundance but low complexity will contribute most heavily to the hybridization. Analysis of the [^{3}H]cDNA hybridization will, therefore, provide insight into the frequency distribution of mRNA sequences in the total population.

Fig. 4 shows the reassociation profile of poly(A)$^+$ mRNA and its complementary [^{3}H]cDNA. Clearly, the hybridization kinetics are complex, extending over 6 log units of R_0t. A computer program essentially analogous to that used by Levy and McCarthy [20] was employed to obtain the best theoretical description of the population distribution. Analysis of the data obtained as best fit suggests a minimum of three kinetic components. As previously described [21], this result indicates a mRNA population composed of three abundance classes. The percentage of [^{3}H]cDNA in each abundance class and the corresponding $R_0t_{½}$ value for that class are presented in Table III.

By comparison of the corrected $R_0t_{½}$ value of the frequency class with that of a known standard (Fig. 4) [21], the sequence complexity of the mRNA population represented in each [^{3}H]cDNA abundance class can be elucidated. Under our conditions of hybridization, a $R_0t_{½}$ value of 6.6×10^{-4} (Fig. 4) was obtained for hybridization of [^{3}H]cDNA complementary to a mixture of rabbit globin α and β mRNA with excess

TABLE II

Sequence complexity of RNA populations by hybridization to ^{3}H-labeled single-copy DNA.

RNA	Saturation value (%)[a]	Corrected saturation value (%)[b]	Complexity (nucleotides)[c]	K_{obs}^{-1} ($M^{-1} \cdot s^{-1}$)	K_{exp}^{-1} ($M^{-1} \cdot s^{-1}$)[d]	F[e]	No. 1350 NT sequences
Total polysomal	30	68	1.6×10^7	2.0×10^{-3}	3.2×10^{-2}	6.3×10^{-2}	11 850
Poly(A)$^+$-polysomal	21	49	1.2×10^7	2.0×10^{-2}	4.2×10^{-2}	4.8×10^{-1}	8 900

[a] The values are the terminal hybridization values described by a least-squares computer solution of the data in Figs. 3A and B. The standard deviations of these solutions were ± 0.69% for total polysomal RNA and ± 0.37% for polysomal poly(A)$^+$-mRNA.

[b] The saturation value was corrected for asymmetric transcription and reactability of the ^{3}H-labeled single-copy DNA = (saturation value × 2)/(reactivity of ^{3}H-labeled single-copy DNA).

[c] Complexity (C_{RNA}) = corrected saturation value × (2.4×10^7), where 2.4×10^7 nucleotide pairs is the complexity of single copy DNA from *T. cruzi* (Table I).

[d] Pseudo-first-order rate constant predicted from an RNA population of a known complexity. The predicted rate is calculated from the relationship:

$$K_{exp} = \frac{5374 \times 200}{C_{RNA}} \times (300/l)^{1/2}$$

where 5374 is the complexity of ϕX174 RNA, 200 $M^{-1} \cdot s^{-1}$ is the pseudo-first-order rate constant for an RNA excess hybridization between ϕX174 RNA and 300 nucleotide tracer RF DNA [31]. L is the mass average length of the *T. cruzi* RNA (1350 NT for both populations).

[e] The fraction of the RNA which is driving the reaction (the most complex RNA in the population) as calculated from the ratio of K_{obs} to K_{exp} [17, 32].

TABLE III

Sequence complexity of *T. cruzi* polysomal poly(A)$^+$-mRNA by hybridization to [^{3}H]cDNA.

Transition No.[a]	Percent [^{3}H]cDNA[b]	$R_0t_{1/2}$	$R_0t_{1/2}$ (if pure)[c]	No. of sequences[d]	Copies per cell[e]
1	14	7×10^{-3}	1.0×10^{-3}	2	2 800
2	36	1.2	0.43	750	20
3	50	18	9.0	15 500	1–2

[a] The transitions refer to the frequency classes defined by computer analysis of the hybridization profile shown in Fig. 4.

[b] Calculated from the data in Fig. 4 as presented in the figure legend. The percentage of [^{3}H]cDNA in each component is obtained by dividing the value observed in Fig. 4 by 0.7, the fraction cDNA hybridized between $R_0t = 0$ and the plateau value in Fig. 4.

[c] $R_0t_{1/2} \times$ % cDNA as in Bishop et al. [21].

[d] Obtained by dividing the $R_0t_{1/2}$ (if pure) by the $R_0t_{1/2}$ (calculated) for an average sequence. The R_0t (calculated) is determined as follows: The rabbit globin mRNA has a complexity of 1320 nucleotides and under our conditions of hybridization, a $R_0t_{1/2}$ value of 6.6×10^{-4}. The average sequence length of a *T. cruzi* mRNA molecule is 1350 nucleotides or 1.02 times the sequence length of the globin. The average lengths of the tracer cDNAs for globin and *T. cruzi* were determined to be 470 and 550 nucleotides, respectively. The tracer length of the *T. cruzi* is, therefore, 1.17 times that of the globin. The $R_0t_{1/2}$ (calculated) correcting for the effect of tracer length [33] is

$$\frac{(6.6 \times 10^{-4}) \times 1.02}{1.17} = 5.8 \times 10^{-4}$$

The complexity standard (globin mRNA: [^{3}H]cDNA) was hybridized in the presence of the same Na^+ concentration as used for the *T. cruzi* mRNA to avoid the application of a differential salt correction factor.

[e] Calculated using using the following parameters. The average amount of polysomal RNA per cell is 8.2 pg (Lanar and Manning, unpublished observation) and the percentage of poly(A)$^+$-RNA is 3.7. The average size of *T. cruzi* mRNA is 1350 nucleotides. The average number of mRNA molecules per cell is calculated to be approximately 39 000. Using this approximation, copies per cell = 39 000 × % [^{3}H]-cDNA)//No. of sequences).

unlabeled globin mRNA. Using this $R_0t_{1/2}$ and a value of 1320 nucleotides for the combined complexity of α and β globin mRNA, the sequence complexity and number of different mRNA sequences present in each of the three mRNA abundancy classes was calculated (Table III). Three salient features of the mRNA population become evident from analysis of these data. First, the total number of different poly(A)$^+$-mRNAs is ca. 16 000 or 1.9 times that observed from the previous saturation hybridization experiments. Since the latter approach measured the expression of only the single-copy DNA sequences, while the [^{3}H]cDNA method includes analysis of all DNA sequences classes, it is apparent that the repetitive DNA sequences are quite likely represented in the mRNA population. This is not surprising in view of previous observations with other organisms [22]. Second, it is also clear that about 50% of the total mass of the mRNA is occupied by only 700–800 different mRNA species, or about 5% of the total mRNA

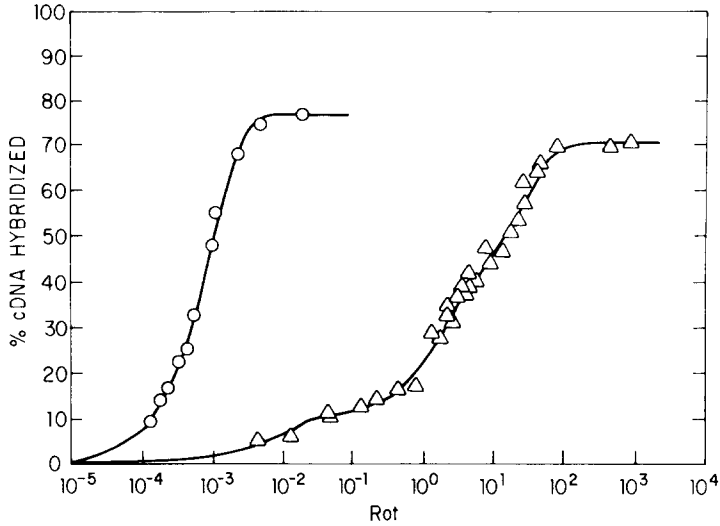

Fig. 4. Hybridization of polysomal poly(A)$^+$-mRNA to [^{3}H]cDNA. [^{3}H]cDNA was reacted with excess poly(A)$^+$-mRNA (△) as described in Materials and Methods. The solid line represents a computer fit of the hybridization values to three pseudo first-order components indicating the presence of three frequency classes. These classes differ in amounts as follows: Of the 70% total hybridization observed, the most abundant class comprises 10% of the RNA–cDNA hybrids, the moderately abundant class contains 25% of the hybrids, the least abundant class comprises 35% of the hybirds (see Table III). Hemoglobin mRNA hybridized in excess to its respective [^{3}H]cDNA (○) was used as a complexity standard. The RMS of this computer solution is 0.004.

complexity. Each of these abundant mRNA species are present in several tens of copies per cell. Third, the vast majority of the different mRNA species (15 500) must be considered a rare class of mRNA with each being present in only 1–2 copies per cell.

DISCUSSION

DNA content of T. cruzi. Previous reports of the amount of DNA in *T. cruzi* have indicated ca. 1.7×10^{-13} g of DNA per cell [5, 23, 24]. Our results, however, indicate that the total DNA content is about 3.3×10^{-13} g, or about two times the above amount. This difference could be explained by the use of different techniques in determination of DNA content. In all previous reports, total cellular DNA was acid extracted and the DNA amount was then determined colorimetrically. Dividing by the cell number in the sample gave the amount of DNA per cell. Our approach differs in the following way: microspectrofluorometry is a technique whereby one focuses directly on the nucleus or kinetoplast of a single-stained cell and measures the amount of fluorescence emitted by that organelle when it is pulsed by a beam of laser light. By this quantitative technique, we measured the nucleus to contain ca. 2.8×10^{-13} g of DNA, and the kinetoplast to contain ca. 5.4×10^{-14} g of DNA for a total of ca. 3.3×10^{-13} g/cell. Furthermore,

by analysis of the hybridization rate of the nuclear single-copy DNA sequences, we determined *T. cruzi* to contain 2.4×10^8 NTP (2.6×10^{-13} g) of DNA/diploid genome. This value is in good agreement with our microspectrofluorometric analysis of the DNA content of the *T. cruzi* nucleus. Most importantly, neither technique is influenced by the inherent inaccuracies present in cell counting procedures or in loss of DNA during extraction. Furthermore, the percentage of total cellular DNA present in the kinetoplast was measured as 16% by microspectrofluorometric analysis, a number comparable to that obtained for kinetoplast DNA by buoyant bandings of nuclear and kinetoplast DNA in a neutral cesium chloride density gradient [5]. The present findings provide strong chemical evidence for the diploidy of *T. cruzi*. In is interesting to note that, recently, Tait [25] presented evidence suggesting that strains of *T. brucei* are diploid, and undergo random mating.

Sequence complexity of T. cruzi *epimastigote mRNA*. Hybridization of total polysomal RNA to ^{3}H-labeled single-copy DNA revealed the complexity of this RNA population to be 1.6×10^7 nucleotides, which corresponds to approximately 11 900 different mRNA molecules of average size 1350 nucleotides. The hybridization of polysomal poly(A)$^+$-mRNA to ^{3}H-labeled single-copy DNA indicates a complexity which is about 75% of that observed for total polysomal RNA (i.e., 1.2×10^7 nucleotides, or 8900 different mRNAs). Thus, the mRNA population in *T. cruzi* is similar to that of several higher eukaryotic organisms [6, 15, 16, 26–28] in that it exhibits a distinct population of nonadenylated mRNAs (ca. 3000) which is not represented in the poly(A)$^+$-mRNA class.

In order to determine the mass distribution of mRNA species in the poly(A)$^+$-population, poly(A)$^+$-mRNA was hybridized to its complementary [^{3}H]cDNA. Analysis of the hybridization profile indicates a low, medium and higher complexity class, each of which contains approximately 2, 750 and 15 500 different mRNA sequences, respectively. The low complexity RNA species are present in about 3000 copies per cell, while the medium and high complexity RNA are present at about 20 and 1–2 copies per cell, respectively. Although we do not know what proteins are encoded by the mRNAs in these classes, it is clear that in *T. brucei* the mRNA that codes for the major variable specific surface antigen (VSSA) is present in a low complexity, high abundance class [29]. Therefore, we are currently investigating whether the mRNAs which code for the major surface antigens on *T. cruzi* are also represented in this low complexity, high abundance class.

The value obtained for the sequence complexity of the poly(A)$^+$-mRNA population by [^{3}H]cDNA–poly(A)$^+$-mRNA hybridization is substantially greater than that observed by hybridization of poly(A)$^+$-mRNA and ^{3}H-labeled single-copy DNA. This difference may in part be due to the presence of mRNA species in the population which are transcribed from repetitive DNA sequences. Such mRNAs would contribute to the total poly(A)$^+$-mRNA complexity as measured by the [^{3}H]cDNA approach, but would be refractory to analysis by hybridization of poly(A)$^+$-mRNA to ^{3}H-labeled single-copy DNA sequences. Since mRNAs complementary to repetitive DNA sequences have been

observed in numerous eukaryotic systems [22], we believe this is also the case in *T. cruzi*. It must, however, also be recognized that even the best theoretical description of a complex reassociation profile contains the inherent difficulty that a significant range in sequence complexity can be obtained within a two-fold range in the root mean square (RMS) of the computer solution to the data. Nevertheless, both the saturation and [^{3}H]cDNA hybridization results show that a large number of different mRNA sequences vary in cellular concentration, and that $>95\%$ of the sequence diversity is represented by sequences present in only a few copies per cell.

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