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CLONING OF GENES FOR ANTIGENICALLY RELEVANT PROTEINS OF TRYPANOSOMA CRUZI

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INTRODUCTION

Trypanosoma cruzi has a complex antigenic make up. Several (glyco)proteins have been identified on their surface membrane (1-2) some of which are specific of a given stage of the parasite life-cycle (3-5). In addition, *T. cruzi* isolates differ in several biological and biochemical properties (6) including antigenic composition (7-8). Analysis of parasite antigens is essential not only to work out practical problems like diagnoses (9-10) and eventually, vaccination (11-12), but also to better understand the immunopathology of Chagas' disease. Since each of the several *T. cruzi* antigens is a minor component of the parasite, recombinant DNA technology may contribute to identify and obtain in sufficient amounts, relevant parasite proteins. In order to be able to answer some of these questions, we isolated and characterized those parasite genes whose products are detected by sera from Chagasic patients (13).

In this paper we summarize previous observations and describe new results on the structural organization of the genes and the antigenic relevance of the fusion proteins.

RESULTS

Isolation of T. cruzi antigen genes and identification of the corresponding parasite proteins.

Screening of a T. cruzi genomic DNA library made in gt11 lambda vector was performed with serum from one chronic Chagasic patient (13). Out of 300,000 recombinant phages, 53 positive clones were obtained. Thirty-two of them were analyzed. Nine different groups were defined according to the sequence homologies of the recombinant clone inserts and to the reactivity of the cloned gene products with selected antibodies (14). That is, clones included in one group have cross-hybridizing inserts and their products react with the antibodies selected from a serum with one of them and, consequently, have similar or identical epitopes. Clones included in each group are shown in Table I. Some groups have a single clone while in others, up to 8 homologous clones were detected. These results may be related, in part, to the particular structure of some of the clone inserts and to the size of the corresponding gene (see below).

To identify the parasite proteins that have antigenic determinants homologous to each of the cloned gene products, we used the antibody selection method (14) followed by reaction of these antibodies with Western blots of protein extracts from axenic culture-epimastigotes and cell-culture trypomastigotes of the RA T. cruzi strain (15). These results are summarized in Table I. Most proteins detected were of high relative molecular weight, the smallest ones being those of clones 2, 13, 36 and 54 (85-90 kDa). Interestingly, several groups have identified 85-90 kDa (glyco)proteins on the surface of T. cruzi related either to parasite internalization (16) or to the binding of fibronectin (17), and also

Table I

Clone N°	Homologous clones	Parasite protein size (kDa)	E or T
1	3-6-33-42-43-44-53	>205	E-T
2	16-20-38	85	T
7	9-12-41	205/200/190/175/165	T
10	45-47	150/140/125	E-T
13	-	85	T
26	15-17	>205	T
30	4-46	205/195/160	E
36	21-27-40-50	85	E-T
54	-	90	T

T. cruzi proteins detected with each recombinant clone. The number of clones per group and size of the parasite protein identified with each recombinant clone are indicated. The column E or T indicates whether proteins have been identified in epimastigotes (E), trypomastigotes (T) or both stages (E-T).

a heat-shock protein (18). Since our four clones whose products are homologous to parasite proteins of 85-90 kDa do not share sequence homologies (unpublished results) with those of the two genes sequenced (18-19) we conclude that there are at least six antigenic proteins within this size range in T. cruzi. The putative mRNA detected with each cloned DNA was described previously (13).

Preliminary immunofluorescence studies (unpublished results) with living parasites and selected antibodies showed that the parasite proteins homologous to the products of clones 1, 2, 10, 13 and 54 are located on the surface. It remains to be studied whether the proteins homologous to the products of the other clones, which are also antigenically active during an infection (see below) are proteins secreted by the parasite.

Genomic organization and structure of the isolated genes

Nuclear DNA of one T. cruzi strain (CA1-65, ref 15) was digested with Bam HI, EcoRI (Fig. 1), Hind III, PstI, HpaII and HaeIII (not shown) and hybridized with ^{32}P-labelled clone inserts. Most clones detected bands compatible with a single gene but for two of them, clones 7 and 13. In the case of clone 7, several bands are detected in PstI, HpaII and HaeIII digest, and interestingly, the overall pattern is the same with the three enzymes but for the differences in the sizes of the bands (Fig. 1B). Clone 13, on the other hand, detected a great number of bands (see also PstI, HpaII and HaeIII digests, Fig. 1C) even under high stringent hybridization conditions. Since a single RNA species was observed with this clone (13), it is possible that the insert of clone 13 has a repetitive sequence which is scattered throughout the T. cruzi genome.

Partial sequencing of the inserts of the nine clones showed that seven of them have an internal tandemly repeated structure. The size of the repeats

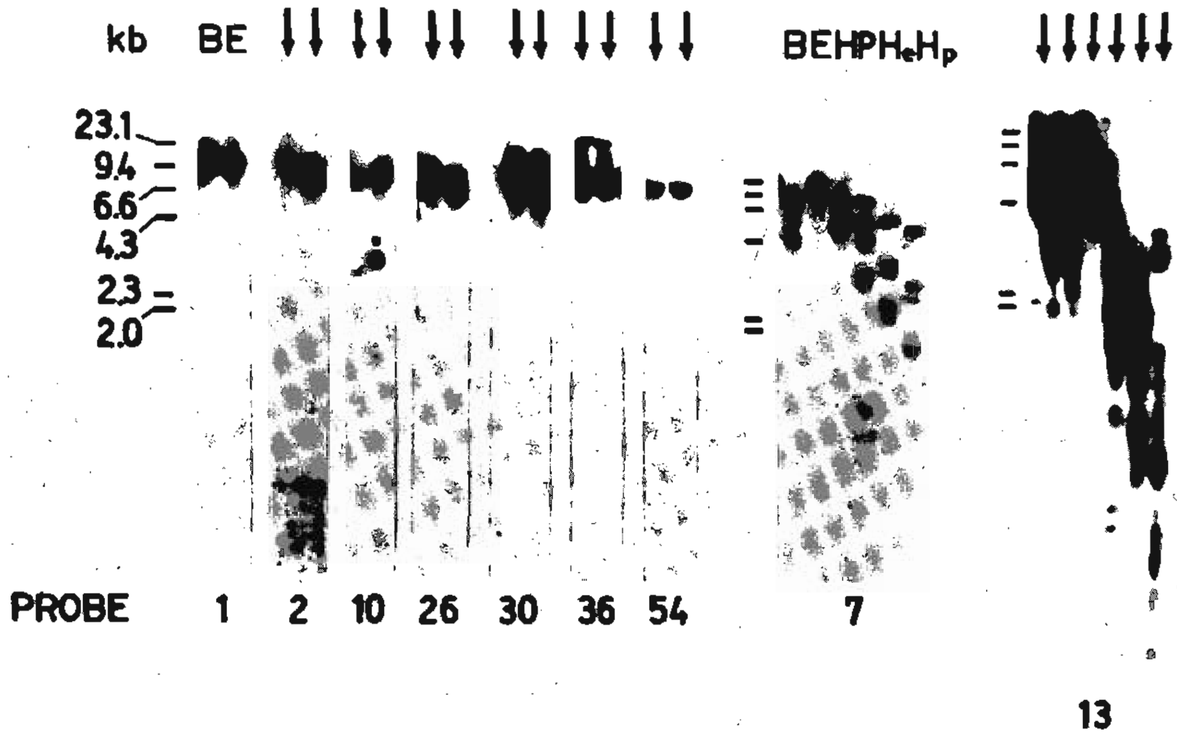

Figure 1.

Genomic organization of the isolated recombinant clones. Nuclear DNA from epimastigotes (CA1 strain) was digested with the indicated restriction endonuclease, run on 0.8% agarose gel, blotted onto nitrocellulose filters and hybridized with the indicated clone inserts. B BamI, E EcoRI, H HindIII, P PstI, He HaeIII and Hp HpaII.

Table II

Clone N°	Repeat size (aa)	N° of repeats sequenced
1	68	7
2	12	18
10	8	19
13	5	31
15 (26)	14	3
30	14	13
36	38	6

Structure of the cloned DNA sequences having internal repeated units. The number of aminoacids (aa) per repeat deduced from the nucleotide sequence and the number of repeats sequenced per clone are shown.

together with the number of units sequenced are shown in Table II. The repeats within each clone are highly conserved and ranged in size from 15 to 204 base pairs. No sequence homology between repeats of different clones was observed. They also differ from those present in the sporozoite and merozoite stages of plasmodium species (20-21) as well as from those _T. brucei_ (22) and _T. cruzi_ (18-19) genes reported. At least in three (1-30-36) of the six clones, the repeated region of the corresponding protein is antigenically active since their inserts are entirely made up of repeated units.

Antigenic relevance of the cloned gene products

In order to know the antigenic relevance of the cloned genes, each gene product was reacted with sera from Chagasic patients obtained in Argentina (23 sera), Brazil (49 sera) and Chile (12 sera) (see ref. 13 for the description of the method used).

Seventy of the 84 sera tested reacted with at least one of the nine cloned gene products (Table III). Most of the sera reacted with more than one clone product and, in this context, it is interesting to note that the products from clones 1, 2, 13 and 30 are enough to detect antibodies in the 70 sera tested but for one from Argentina and one from Brazil. Sera from Argentina and Chile detected in a higher proportion the products from clones 1, 2 and 30, while sera from Brazil detected mainly those from clones 1, 2, 13 and 36 (Table IV). Whether these variations are due to differences in _T. cruzi_ strains and/or are of importance from the immunopathological point of view of Chagas' disease is still a matter of speculation.

Fourteen sera failed to react with any of the nine clone gene products (Table III). Some of these sera are now being used to screen the genomic DNA

Table III

Disease and control sera	Country of origin	Number of sera tested	Number of sera positive with one or more cloned gene product	Percentage
Chagas	Brazil	49	41	84
Chagas	Argentina	23	20	87
Chagas	Chile	12	9	75
Total Chagas		84	70	83
Leishmaniasis (Kala-Azar)	Brazil	21	0	0
Toxoplasmosis	Argentina	9	0	0
Controls	Brazil	14	0	0
Controls	Argentina	23	2	9

Reactivity of sera from Chagas, Leishmaniasis, Toxoplasmosis and controls with cloned gene products.

library in order to know whether they recognize a different set of parasite antigens.

Table IV

Country	No. of sera tested	Number of sera positive with each clone product								
		1	2	7	10	13	26	30	36	54
Argentina	20	16(80%)	15(75%)	6(30%)	5(25%)	9(45%)	9(45%)	18(90%)	7(35%)	3(15%)
Brazil	41	24(59%)	25(61%)	1(2%)	2(5%)	17(41%)	3(7%)	12(29%)	16(39%)	2(5%)
Chile	9	6(67%)	7(78%)	0	0	1(11%)	0	4(44%)	1(11%)	0
Total	70	46(66%)	47(67%)	7(10%)	7(10%)	27(39%)	12(17%)	34(49%)	24(34%)	5(7%)

Reactivity of sera from chronic Chagasic patients with individual products from recombinant clones. The number of sera (per country) reacting with each recombinant clone product are indicated as well as the total number of positive sera tested (see text).

Cloned genes products for the diagnosis of Chagas' disease

Current serological methods make use of whole parasites or parasite fractions (9). These heterogeneous populations of antigens give rise to false positives with control sera as well as with visceral Leishmaniasis (23). If individual proteins are to be used for diagnosis, they should not unspecifically react with control sera and with sera from persons affected with other parasitic diseases. In order to test this, we reacted the nine protein products with 23 control sera from Argentina, 14 control sera from Brazil, 9 sera from Argentina cases of Toxoplasmosis and 21 cases of visceral Leishmaniasis from Brazil (Table

III). All the above sera were negative with the nine proteins, except for two control sera from Argentina that reacted with some of the cloned gene products. Since these two sera also reacted with whole T. cruzi parasites, we consider likely that they are indeed derived from infected persons and were either not well diagnosed or mixed up with other control sera.

CONCLUSIONS AND PROSPECTS

Nine cloned genes for T. cruzi antigens have been identified. They code for parasite antigens ranging in size from 85 to larger than 205 kDa. Their antigenic relevance is shown by the fact that some of them reacted with most sera from Chagasic patients obtained in different geographical regions (Table III and IV). Since these fusion proteins do not react with control sera nor with sera from cases of Leishmaniasis and Toxoplasmosis, we consider some of them good candidates to be used for the serological diagnosis of Chagas' disease. However, it is still necessary to isolate other gene products in order to be able to detect sera from all Chagasic patients (see Table III).

The results in Table IV show that antibodies to some of the cloned gene products (No. 1, 2, 13, 30 and 36) are more frequently represented among sera from Chagasic patients. Interestingly, antibodies to some proteins (No. 26 and 30) are frequently observed in sera obtained in Argentina but not in those sera from Brazil. Further work is necessary to know whether the above differences can be related to the variations in the antigenic make-up of parasites from different geographical regions and/or to the clinical forms of the disease.

Partial sequencing of the nine cloned inserts showed that seven of them have an internal repeated structure, the size of the repeat being from 5 to 68 aminoacids in the corresponding protein. In the case of malaria, proteins with a

repeated structure are essential for parasite survival since they determine the binding domain that interacts with the host cell (21,24). Antibodies against the repeated sequence seem to be effective in experimental vaccination trials (20). Given the fact that in at least three of the seven T. cruzi cloned gene products the repeated unit is the region of the molecule antigenically active (see above), further work on the possibilities of these antigens as vaccines is desirable.

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