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The *Trypanosoma cruzi* Genome Initiative

The *Trypanosoma cruzi* Genome Consortium*

An initiative was launched in 1994 by the Special Programme for Research and Training in Tropical Diseases (TDR) of the WHO to analyse the genomes of the parasites Filaria, Schistosoma, Leishmania, Trypanosoma brucei *and* Trypanosoma cruzi. *Five networks were established through wide publicity, holding meetings of key laboratories and developing proposals which were then reviewed by the Steering Committee of Strategic Research for financial support. The aim of the Programme was to use the platform of these networks to: (1) train scientists from tropical disease-endemic countries; (2) transfer technology and share material and expertise, thereby reducing costs and increasing efficiency; and (3) provide an information system that is accessible globally as soon as the results become available. The initial target was to produce a low-resolution genome map for each of the parasites, but it soon became evident that by using rapidly developing technologies, it might be feasible to complete DNA-sequence analysis for some of the parasites in the next decade, as discussed here by Alberto Carlos Frasch and colleagues, with particular focus on the* T. cruzi *genome initiative.*

In the short period of time since the TDR/WHO Parasite Genome Initiative was set in motion, the progress (both in terms of scientific achievement and collaborative activity, particularly participation of laboratories from endemic countries) must be considered as highly successful. All networks fulfilled an important task in constructing parasite stage-specific cDNA and large fragment genome libraries. Thousands of ESTs (expressed sequence tags) and chromosome markers have been obtained, and the process of chromosome mapping initiated. Significant expansion of these activities is expected during the next two years, in the hope that, with the introduction of high-precision robotics, the genome of the representative parasites will be sequenced within the next five years. This progress will be detailed below for the *Trypanosoma cruzi* Genome Initiative. The laboratories and scientists involved are listed in Box 1. The overall target objectives of the TDR/WHO Parasite Genome Initiative for the next ten years of research, and the network coordinators are shown in Box 2. The relevance of the *T. cruzi* Genome Initiative for the control of Chagas disease, and some of the problems to be solved to achieve these goals, are dealt with in Boxes 3 and 4.

CL Brener as the reference strain

A striking feature of *T. cruzi* is its heterogeneity in relation to biological properties. The parasite has a broad host range, infecting a large variety of mammals and several species of insect vectors. Differences in growth rates, infectivity, tissue tropism and susceptibility to chemotherapeutic drugs have been reported in parasite isolates. In humans, a broad spectrum of clinical presentations in Chagas disease is observed, possibly reflecting the heterogeneity among *T. cruzi* isolates and/or genetic differences in the immune response of the host[1].

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The genetic variability of the parasite has been widely documented by analysis of electrophoretic patterns of isoenzymes, restriction endonuclease digestion profiles of kinetoplast DNA (kDNA) minicircles, DNA fingerprints, randomly amplified polymorphic DNA (RAPD) analysis and molecular karyotypes. Several pieces of evidence suggest that the present genetic and biological diversity of this parasite is the result of the independent evolution of multiple clonal lines that propagate with little or no genetic exchange[2].

As a consequence of the above considerations, the participants of the *T. cruzi* network present in the Trypanosomatid Genome Planning Meeting held in Rio de Janeiro in April 1994, decided to select one reference clone (CL Brener) to be used by all members. This clone derives from the CL strain that presents all the important characteristics of *T. cruzi*. It was isolated from *Triatoma infestans*, a strictly domiciliary vector. It differentiates in liquid medium, infects cell monolayers, shows preferential parasitism of heart and muscle cells, presents defined parasitaemia curves and mortality in mice, shows a clear acute phase in accidentally infected humans, and is highly susceptible to drugs used clinically to treat Chagas disease. Clone CL Brener was recently isolated by Prof. Z. Brener and M.E.S. Pereira (Instituto de Pesquisas René Rachou, Fiocruz, Belo Horizonte) from blood of chronic-phase mice infected with the CL parental strain. A Reference Laboratory (B. Zingales, e-mail: bszodnas @quim.iq.usp.br), at the University of São Paulo, Brazil, was chosen for maintenance, storage and distribution of clone CL Brener to members of the *T. cruzi* Genome Project. At present, this organism has been sent to laboratories in Argentina, Brazil, Chile, France, Mexico, Paraguay, Spain, Uruguay, UK, USA and Venezuela. The tasks attributed to the Reference Laboratory include defining the biological characteristics of CL Brener clone: differentiation conditions; infectivity in mice and in mammalian cells grown *in vitro*, and susceptibility to benznidazole, nifurtimox, allopurinol and ketoconazole. In addition, the molecular typing of the CL Brener clone is being performed by isoenzymatic profiles, schizodeme, RAPD and DNA fingerprinting analyses, and by sequencing of a ribosomal RNA gene. Its genetic stability under prolonged culture conditions is also under investigation.

Box 1. Participating Laboratories of the *Trypanosoma cruzi* Genome Consortium[a]

Centro de Biología Molecular, Universidad Autónoma de Madrid, Madrid, Spain: *Carlos Alonso, José María Requena.*

Fondation Jean Dausset – CEPH (Centre d'Etude de Polimorphisme Humain), Paris, France: *Daniel Cohen, Denis Le Paslier, Marcial Saumier, Virginie Perrot.*

Unité de Parasitologie Experimentale, Institut Pasteur, Paris, France: *Mireille Hontebeyrie-Joskowicz, Paola Minoprio.*

Escola Paulista de Medicina. UNIFESP, São Paulo, Brazil: *Jose Franco da Silveira, Maria Isabel Cano, Marçia R. Santos.*

Departamento de Bioquímica e Biología Molecular, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil: *Wim Degrave, Adeilton Brandao, Antonio de Miranda, Antonio Gonçalves.*

Centro de Pesquisas René Rachou, FIOCRUZ, Belo Horizonte, Brazil: *Maria Elizabeth S. Pereira.*

Instituto de Medicina Tropical, São Paulo, Brazil: *Eufrosina S. Umezawa, Kátia A. Almeida.*

Departamento de Bioquímica e Imunología, Universidade Federal de Minas Gerais, Belo Horizonte *Riva P. Oliveira, Andrea Macedo.*

Instituto de Investigaciones en Genética y Biología Molecular (INGEBI): Buenos Aires, Argentina: *Mariano Levin, Silvia Brandariz, Inés Ferrari, Alejandro Schijman, Cecilia Medrano, Martin Vasquez, Hernán Lorenzi, Claudia Ben-Dov.*

Instituto de Parasitología y Biomedicina, Granada, Spain: *Antonio Gonzalez, Arantxa Cortes.*

Instituto de Biofísica Carlos Chagas Filho, UFRJ, Rio de Janeiro, Brazil: *Edson Rondinelli, Turan Urmenyi.*

Universidad Central de Venezuela, Caracas, Venezuela: *Jose Luis Ramirez.*

Universidad Simón Bolívar, Caracas, Venezuela: *Rafael Rangel-Aldao.*

Instituto de Química da Universidade de São Paulo, São Paulo, Brazil: *Bianca Zingales, Arthur Gruber, Ricardo P. do Souto.*

Instituto Nacional de Chagas 'Dr Mario Fatala Chaben', Buenos Aires, Argentina: *Andrés M. Ruiz, Jacqueline Búa, Esteban Bontempi, Betina Porcel, Carlos Pravia, Gabriela García.*

Instituto de Investigaciones Bioquímicas 'Luis F. Leloir'. Fundación Campomar/ CONICET/ Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina: *Alberto Carlos Frasch, Juan José Cazzulo, Daniel Sánchez, Guillermo Uemura, Marcelo Guerin.*

Instituto de Investigaciones Biotecnológicas, Universidad Nacional de General San Martín, Villa Ballester, Argentina: *Alberto Carlos Frasch, Rodolfo A. Ugalde.*

Department of Medical Parasitology, London School of Hygiene and Tropical Medicine. Keppel Street, London, UK WC1E 7HT: *John M. Kelly, Angeles Mondragon.*

Department of Microbiology and Immunology, University of Memphis, Memphis, TN 38163, USA: *John Swindle.*

Molecular-Genetic Genome Analysis, Deutsches Krebsforschungszentrum (DKFZ), Heidelberg, Germany: *Jörg D. Hoheisel, Jens Hanke.*

Department of Medical Genetics, Biomedical Center, University of Uppsala, Uppsala, Sweden: *Ulf Pettersson, Lena Åslund, Björn Andersson.*

TDR/WHO Secretariat: UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases, World Health Organization, Geneva, Switzerland: *Farrokh Modabber, Boris Dobrokhotov.*

[a]Meetings involving representatives of these laboratories are held whenever possible: for example, at the 1995 Woods Hole Molecular Parasitology Meeting (Woods Hole, Massachussetts, USA, September, 1995); at the XXII Annual Meeting on Basic Research in Chagas Disease (Caxambu, Minas Gerais, Brazil, November, 1995); at the International Symposium on 'New Alternatives for the Study of the *Trypanosoma cruzi* Genome. (Instituto Nacional de Chagas 'Dr Mario Fatala Chaben', Buenos Aires, Argentina, 14 November 1995), at the International Symposium 'The Human Genome Project and Parasite Genome Projects: Progress and Perspectives' (INGEBI, Buenos Aires, Argentina, 13 14 November 1995), and at the International Training Course on 'Parasite Genome Projects: Strategies and Methods' (ICRO-UNESCO, WHO/TDR, ONUDI, CYTED, LABBIO; INGEBI Buenos Aires, Argentina, 13–24 November 1995).

The CL Brener nuclear karyotype and gene mapping

The genetic material of *T. cruzi* is organized in small chromosomes that are poorly condensed during cell division, making its analysis by conventional cytogenetics impossible. Size fractionation of chromosomal bands by pulsed field gel electrophoresis (PFGE) and hybridization of different DNA probes

Box 2. The TDR/WHO-sponsored Parasite Genome Initiative Networks

- The *Filaria* genome network will focus on *Brugia malayi*. High quality cDNA libraries will be constructed and evaluated for all developmental stages of *B. malayi*. The known map of *Caenorhabditis elegans* will be used extensively to identify numerous genes, thereby facilitating the work of the network. (Co-ordinated by: Dr Steven Williams, Department of Biological Sciences, Clark Science Center, Smith College, Northampton, MA 01063, USA. Tel: +1 413 585 3826, Fax: +1 413 585 3786, e-mail: Swilliams @smith.smith.edu).
- The *Leishmania* genome network will focus on a single *L. major* strain (Friedlin). An international consortium has been established to sequence the entire genome. The cosmid library and/or high density filters prepared from it can be obtained from a central laboratory. The resource also includes EST sequence information on many new genes, clones for which can be obtained by any parties interested in their development as drug targets or vaccines. (Co-ordinated by: Prof. Jenefer M. Blackwell, Department of Medicine, University of Cambridge, Addenbrooke's Hospital, Hills Road, Cambridge, UK CB2 2QQ. Tel: +44 1223 636 8636, Fax: +44 1223 436 5389, e-mail: JMB37@uk.ac.cam.phx@umc).
- The *Trypanosoma brucei* genome network will not focus on any particular cloned isolate because a substantial number of markers are available which can be used for physical mapping and have been developed using different isolates. However, genomic libraries are needed and will be constructed by a selected laboratory. This will act as a trypanosomatid reference centre to maintain large DNA libraries for distribution (Co-ordinated by: Prof. John Donelson, Howard Hughes Medical Institute Research Laboratories, Department of Biochemistry, 300 D. Eckstein Medical Research Building, College of Medicine, University of Iowa, Iowa City, IA 52242, USA. Tel: +1 319 335 7889, Fax: +1 319 335 6764, e-mail: jedonels@vaxa.weeg.uiowa.edu).
- The *Trypanosoma cruzi* genome network has chosen a single, well-developed CL Brener clone from Brazil. The main activities include the construction of genomic libraries from different stages in the parasite. Small sections will be mapped initially with the development of the entire map in view, using contig libraries. (Co-ordinated by: Prof. Alberto Carlos Frasch, Fundación Campomar, Instituto de Investigaciones Bioquímicas, Av. Patricias Argentinas 435, 1405 Buenos Aires, Argentina. Tel: +54 1 863 4011 to 4019, Fax: +54 1 865 2246, e-mail: cfrasch.fc@iris.iib.uba.ar).
- The *Schistosoma* genome network selected *S. mansoni* and *S. japonicum*. Priority has been given to four objectives towards mapping of the *Schistosoma* genome: (1) Resource development: stage-specific cDNA libraries are available, but large fragment genomic libraries are needed; (2) gene discovery, including identification of stage-specific genes; (3) low-resolution physical mapping; and (4) development of the genome databank. (Co-ordinated by: Prof. Mette Strand, Department of Pharmacology and Molecular Sciences, The Johns Hopkins University, School of Medicine, 401 Hunterian, 725 North Wolfe Street, Baltimore, MD 21205, USA. Tel: +1 410 955 8477, Fax: +1 410 955 1894, e-mail: Mette.Strand@qmail.bs.jhu.edu).

have been used to establish the molecular karyotype of several strains and clones of *T. cruzi*. The nuclear genome of *T. cruzi* is highly plastic, and the size and number of chromosomal bands vary among different strains and clones of this parasite (reviewed in Ref. 3).

The molecular karyotype of the clone CL Brener was analyzed by PFGE, using either single-run conditions[4] (e-mail: franco.dmip@epm.br) or three different overlapping separations[5] (e-mail:lena.aslund@ medgen.uu.se). In both cases the technique allowed visualization of chromosomal bands ranging from 0.45 to 4.0 Mbp; by using different sets of PFGE conditions (able to resolve more specifically in the lower, middle and higher size range), up to 42 distinct bands could be detected[5]. The same chromosomal pattern was obtained with the parental CL strain[4]. When the CA-I/72 and Sylvio X10/7 clones were compared to CL Brener, substantially different patterns were found, the chromosomes in CL Brener being, in general, larger than the homologous ones in the other clones[5]. The intensity of the ethidium bromide fluorescence after staining of the PFGE gel was not evenly distributed along the chromosomal bands, indicating comigration of two or more chromosomes, or aneuploidy[4]. However, the ethidium bromide-stained banding pattern showed good correlation with the hybridization pattern obtained with a *T. brucei* telomeric probe, suggesting a more or less even distribution of telomeric sequences[4]. Both studies suggested that most of the chromosomal bands represent pairwise homologous chromosomes; the maximal number of chromosomes in the CL Brener clone was estimated as at least 64 (Refs 4, 5). The total nuclear content of the clone CL Brener was estimated[4] to be approximately 87 Mbp.

Chromosomal location of *T. cruzi* highly and middle repetitive DNA sequences, RNA and protein encoding genes was carried out by hybridization of chromoblots with homologous probes[4]. Highly repetitive sequences account for about 9% of the nuclear genome[9]. Two middle interspersed repetitive DNA sequences, C6 and SIRE (short interspersed reiterated element)[7–9], were mapped to all chromosomal bands, while a minisatellite and a retrotransposon-like sequence were mapped to 11 and 10 chromosomal bands. Members of the ribosomal P2beta gene family and gp85 gene family (gp90, gp85, gp82) hybridized with chromosomal bands 6 and 10–13, respectively[4]. Some genes have unique chromosomal locations, and can be used for the identification of single chromosomes. Several genes were mapped at neighbouring chromosomal bands, as was the case for the 2.3 and 2.6 Mbp bands, apparently representing homologous chromosomes (M.R. Santos and J.F. Silveira, unpublished). Furthermore, single-stranded conformational analysis has been used to generate a set of STSs that differentiate alleles that map to different chromosomes[10].

Cano *et al.*[4] confirmed the physical linkage of several antigen genes (stage-specific surface antigen genes; B12 and B13 repetitive antigens) by restriction analysis and sequencing of genomic clones carrying linked copies. Mapping efforts proceed using probes generated from cDNA libraries, inter-SIRE and SIRE-bubble PCR (polymerase chain reaction), and whole chromosome labeling.

In the study by Henriksson *et al.*[5], chromosome identification was performed by hybridization with 35 different probes[11], 30 of which detected single chromosomes. The chromosome-specific probes identified

between 26 and 31 chromosomal bands in the three cloned stocks used, corresponding to 20 unique chromosomes in CL Brener and 19 in CA-I/72 and Sylvio X10/7. Considering the DNA content of the parasite, it was predicted that the markers recognize at least half of all *T. cruzi* chromosomes. A majority of identified chromosomes showed large differences in size among different strains, in some cases by up to 50%. Several of the markers showed linkage, and nine different linkage groups were identified, each comprising 2–4 markers. The linkage between the markers was maintained in eight of the nine linkage groups when a panel comprising 26 different *T. cruzi* strains representing major *T. cruzi* populations was tested. One linkage group was found to be maintained in some strains but not in others. This result shows that chromosomal rearrangements occur in the *T. cruzi* genome, albeit with a low frequency. Repetitive DNA, both non-coding and (in one case) coding, was more abundant in the cloned stock CL Brener than in CA-I/72 or Sylvio X10/7 (Ref. 5).

Box 3. North–South Partnership Networks[a]

As a result of a recent restructuring, the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR) decided that its Strategic Research component would support research in three well-defined areas: (1) molecular entomology, (2) parasite genome; and (3) pathogenesis.

On 14–15 April 1994, during a meeting jointly sponsored by TDR and the Oswaldo Cruz Foundation, at FIOCRUZ's headquarters in Rio de Janeiro, Brazil, scientists working with *Leishmania* and with American and African trypanosomes laid down the foundations for the initiative now known as the 'Parasite Genome Initiative' of TDR.

The review of the first year of development of the funded projects, carried out at the Meeting of the Steering Committees on Strategic Research, TDR, in Geneva, 25–29 September 1995, was extremely positive. An excellent organization of research networks has been established involving scientists and institutions from developed and developing countries working in close partnership and focusing on the selected genomes. The initial steps of cloning, characterization and mapping are already yielding contig sequences, STSs and ESTs; sequence databases are being organized at each network using compatible software; training of human resources and technology transfer in this extremely active field is receiving a high priority. This initial success of TDR's Parasite Genome Initiative, however, does not mean that the future of the project will be without difficulties. The challenges facing investigators, institutions and policy-makers are formidable and vary in nature: (1) an economic issue is to be considered – the sequencing of millions of base pairs will require millions of dollars; (2) a technological dilemma has already surfaced, ie. in view of the paradigm shift that is taking place in sequencing projects – will a network of small labs be as efficient as centralized facilities planned for high throughput and mass sequencing?; (3) a policy issue will soon knock at the door of the endemic country laboratories – why invest in a high-tech, basic research project, when there are so many other priorities in health and in science and technology in the Third World?

These are not minor questions, and the participants of the Parasite Genome Initiative, at all levels, should address them carefully if the project is to achieve its goals in time. Governments, decision-makers, sponsors, international organizations, national authorities, institution directors and scientists from developing countries should share the enthusiasm and support the arguments of those who shaped and are conducting this unique initiative: (1) the developing countries have to cope with the breathtaking advances of basic biological sciences, and this is one of the very few opportunities available to join a long-term, far-reaching project; (2) the TDR 'umbrella' gives credibility and momentum to the Programme, catalyzing the participation of advanced and endemic-country laboratories in global research networks, thus assuring real technology transfer between partner laboratories; (3) no matter what final strategy is adopted (networks of small laboratories and/or centralized facilities) efficient structures should be implemented in developing countries to allow them to work competitively in the field; (4) financing of the project should come from several sources, including the developing country themselves, since the outcomes of the research will contribute to the future improvement of their health conditions, and will foster their scientific and technological development in a fundamental way.

The TDR Parasite Genome Initiative constitutes a tough challenge in the field of tropical disease research. However, if properly conducted, it will open a real window of opportunity towards bringing advanced science and technology, as well as trained human resources, to developing countries.

[a]Box 3 text is provided by Carlos M. Morel, president of the Fundaçao Oswaldo Cruz (FIOCRUZ), and researcher at the Department of Biochemistry and Molecular Biology, Instituto Oswaldo Cruz, FIOCRUZ, Av. Brasil 4365, Manguinhos, Rio de Janeiro, RJ 21045-900, Brazil (morel@dcc001.cict.fiocruz.br)

Construction of a genomic library in the cosmid shuttle vector pcosTL

A cosmid shuttle vector, pcosTL, facilitates the transfection-mediated introduction of large DNA fragments into *T. cruzi*[12]. In transfected parasites, recombinant cosmids replicate extrachromosomally in multiple copies and genes within the inserts can be expressed at high levels. Cosmid DNA can be rescued after transformation of *E. coli* with total cellular DNA. Because of its applicability both to mapping and to functional studies, we have used this vector as part of the genome project to construct a genomic library with DNA from CL Brener clone.

The library of 60 000 clones was constructed using size-fractionated DNA (20–50 kb) which was obtained after partial Sau3a digestion. Using the automated facilities at the Imperial Cancer Research Fund Laboratories, London, 7200 clones were picked into 384-well microplates and high density colony filters produced (initially 12 copies). To assess the quality of the library, these filters have been hybridized with a number of probes including superoxide dismutase, a thiol-specific antioxidant homologue, group I and group III members of the *trans*-sialidase superfamily, mucin, cruzipain, trypanothione reductase, cyclophilin and adenylate cyclase. Each of the probes yielded positives. Subsequent analysis of several of these clones revealed that the inserts faithfully reflected the structure of genomic DNA. The mucin-like glycoprotein genes have been found to be linked to members of the *trans*-sialidase superfamily at multiple sites in the genome[13]. The filters are now freely available to interested workers for screening (e-mail: j.m.kelly@lshtm.ac.uk). Probes can also be sent to London, UK, and DNA from any positives obtained will be returned.

Box 4. The *Trypanosoma cruzi* Genome Project and the Control of Chagas Disease[a]

The research of Elsa Leonor Segura at the Instituto Nacional de Chagas 'Dr Mario Fatala Chaben' started in 1970 with an analysis of different subcellular fractions of the parasite in terms of eliciting an immunoprotective response from the host. After some years, our efforts centered in the flagellar fraction, isolated from the culture epimastigote stage, and later on the effects of isolated antigenic macromolecules. The advent of the approaches of modern Molecular Biology allowed us, as well as many other researchers in the field, to work with pure recombinant antigens. Yet, even in the very best experimental protocols, complete sterile immunity was not achieved, although very promising results were obtained, both in terms of reduced parasitaemia and increased survival of animals.

Our efforts turned, then, into a search for a better understanding of the behaviour of the parasite and the disease in Nature, to estimate the magnitude of the natural parasite challenge and to improve strategies for the control of natural, vectorial, transmission of Chagas disease. Our field research in rural areas of the endemic zone of Argentina, in 1975–1985, showed a high domiciliary infestation with *Triatoma infestans*, correlated with a high prevalence of *T. cruzi* in the bugs, and a high infection rate both in humans and in domestic dogs. These findings led us to a reformulation of our research priorities, because we felt that, even if an effective vaccine could be obtained at that time, the epidemiological situation might make it of little value in the field. Our efforts then focused on ways to improve the conditions for the control of Chagas disease, both through an aggressive de-insectation campaign with effective insecticides, and through the health education of the population in the endemic areas. This approach, which has left both entomological surveillance and de-insectation (through the use of insecticide canisters) in the hands of the rural population, together with Government conducted campaigns, has resulted in a considerable decrease in both the incidence and the prevalence of Chagasic infection. Overall prevalence of Chagas disease in Argentina has decreased from about 10.4% in the early surveys on young soldiers in the 1960s, to about 6% at present. Moreover, the Summer of 1996 has been the first recorded one without acute cases reported in the Argentinian Province of Santiago del Estero, which used to be the most infected area in the country, and where, until 1993, an average of 25 acute cases per Summer were reported. The situation is evolving in a similar manner in other Latin American countries, thanks to the national efforts coordinated by PAHO (Pan American Health Organization) and the TDR/WHO.

The time is now ripe for a renewed attack against the parasite itself, both through the development of immunoprotective reagents and of new chemotherapeutic agents. For this purpose, the comprehensive information on the biochemistry and molecular biology of the parasite (which will be the result of the Genome Project) will be of inestimable value. We can envisage a time, in the near future, where the information stored in the databases on the parasite will allow us to pick up better antigens for both immunoprotection and diagnosis, and new enzymes that will be suitable targets for chemotherapy. The association of these basic approaches with the wealth of field expertise already attained in the epidemiological studies and control campaigns, is likely to result, within our lifetime, in the final eradication of Chagas disease.

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Construction and analysis of a representative library in a modified Lawrist7 cosmid vector

For the generation of a contiguous cosmid coverage of the genome of *T. cruzi* as a preparation for a large-scale sequence analysis, a cosmid library of 36864 individual, primary clones was generated from total genomic DNA of the reference clone CL Brener both by partial digestion with MboI and from sheared DNA[14]. For cloning, a modified version of cosmid vector Lawrist7 (Ref. 15) that simplifies analyses such as restriction mapping was used. The Lawrist series has been found in several projects (eg. sequencing of the yeast and *Arabidopsis* genomes), to be an extremely convenient cosmid vector with regard to sequence analysis of the cloned DNA. The library's representation is about 25 genome equivalents, assuming a size of 55 Mb per haploid *T. cruzi* genome. From prior experience, such a degree of redundancy was found reasonable for continuous physical mapping, and is essential for resolving problematic areas. No chimerism of inserts could be detected by a stringent assay utilizing the presence of the highly repetitive 196 bp satellite unit as measure. The co-linearity between cosmids and genomic DNA was verified by comparing the restriction patterns of cosmids with the restriction fragments identified by hybridizing the respective clones to Southern blots of the genomic DNA digests. Also, hybridizations to separated *T. cruzi* chromosomes were carried out as a quality check. Gridded on to two nylon filters, the entire library was analyzed using a variety of probes confirming the good representation of the genome in the library. In a reference system, library filters and clones are being made available to interested parties (e-mail: J.Hoheisel@DKFZ-Heidelberg.DE). Clone filters have been used for more than 60 subsequent hybridization experiments[14]. Mapping is under way following the strategy that was used successfully to dissect completely the 14 Mb genome of *Schizosaccharomyces pombe*[16]. Cosmids and cDNA are being used as probes for hybridization with filters containing the entire library. cDNAs are useful probes not only for sorting the cosmids, but also for locating the relevant genes on to the map. Contig construction has been started for chromosomes 1 (Uppsala), 2 (Buenos Aires) and 3 and 4 (Heidelberg) (chromosome numbers as in Ref. 5). Filters containing the BAC (bacterial artificial chromosome) library constructed by John Swindle (see below) are being made, and will be available on request.

Construction of Yeast Artificial Chromosome and Bacterial Artificial Chromosome libraries

In order to start physical mapping of the *T. cruzi* genome, a CL Brener YAC (yeast artificial chromosome) library was constructed. It contains 3000 YACs, with a mean insert size of 365 kb; the library contains more than 10 genome equivalents. A portion of the library has been characterized to test representativity and depth. Particular emphasis has been given to the use of repetitive elements, such as SIRE, for YAC characterization. PCR screening protocols of pooled

arrays of YACs have been used to identify YACs containing housekeeping genes, and genes encoding relevant antigens, including Gp90, 85 and 82, H49 and MIP. Two *T. cruzi* BAC libraries have been also constructed; they have the main advantage that large fragments of DNA can be cloned into an *E. coli* based system. Besides, unlike cosmids, the copy number per cell is strictly controlled such that recombination events leading to rearrangements will be substantially reduced[17,18]. BAC I library contains 9000 clones and BAC II library 4000 clones, with a mean insert size of 20 and 30 kb, respectively. Aiming to improve the mean insert size, a third library is under construction (e-mail: mlevin@proteus.dna.uba.ar).

Contig assembly has begun around several well-characterized loci, such as H49-JL7, B11-B12-B13 and MIP. Simultaneously, a modified version of the Genome Sequence Sampling strategy[19] was developed to support the construction of the physical map of the parasite genome. Isolated chromosomes (I, III and VII, Ref. 4) are being used as probes to hybridize high-density filter membranes to organize YACs and BACs into chromosome-specific subsets. Chromosome specific markers (STSs, sequence-tagged sites, from a set of SIRE-associated sites) are being used to confirm the chromosome specificity of the selected cloned fragments. Since BAC DNA is easy to prepare, and probes from the ends of the cloned insert can be readily made and sequenced, such end probes together with sequence data will be used to assemble chromosome specific contigs. YACs will be used to connect established BAC contigs to yield a continuous physical map of each chromosome[20].

Another BAC library of the CL Brener clone was constructed (e-mail: jswindle@u.washington.edu), yielding clones covering DNA of about four times the length of the parasite genome, with an average size of 50 kb. This group is now in the process of making new libraries with increased insert sizes and with larger overall clone yields.

Construction of cDNA libraries and EST sequencing

A cDNA library from CL Brener epimastigotes using the pSport 1 plasmid (GibcoBRL) was constructed. Poly(A) + RNA from epimastigotes was used to generate a library containing about 320000 clones, which corresponded to a transformation efficiency of 6.4×10^6 transformants per microgram of vector DNA. This number of clones is presumably enough to represent the whole population of expressed sequences. The average insert size was approximately 1.0 kb, ranging from 190 bp to 3.4 kb, as estimated from restriction analysis of 98 randomly chosen clones. Thus far, more than 300 clones have been sequenced, yielding on average 350 bp of useful data per run on a ABI 373A automated sequencer.

About 50% of the sequences did not match with any entry in GenBank/dbEST, the remainder showing homology to previously submitted sequences from other organisms (35%) or from *T. cruzi* (15%). Detailed computer analysis is being performed, and submission to GenBank's EST division is in preparation. In addition, the preliminary identification of chromosome-specific ESTs was started using a random selection of clones, which were probed with isolated chromosomes. The entire chromosomal band number XVI (2.3 Mb) was used as a probe, recognizing two out of 96 purified clones and 42 clones in 20000 colonies of the library. Further experiments are being performed to determine the chromosomal location of the clones recognized and to exclude the potential presence of repetitive sequences.

To minimize the sequencing of already known *T. cruzi* genes when searching for ESTs, the library is being screened with previously identified and cloned genes using probes supplied by several investigators or from the EST sequencing effort. In order to extend the number of available CL Brener cDNA libraries, large numbers of metacyclic trypomastigotes are being produced for poly(A) + RNA isolation and construction of the corresponding cDNA library. Furthermore, both the epimastigote and metacyclic trypomastigote libraries will be 'normalized'. Normalized cDNA libraries are made in such a way as to reduce the representation of abundant mRNA species and increase that of rare species. Thus, the frequency of clones does not reflect the relative abundance of mRNA species in the RNA population, and the library would presumably contain a greater number of different expressed sequences. The normalization procedure to be used involves amplification of single-stranded DNA clones and selection using reassociation kinetics[21] (e-mail: edrondin@ibccf.biof.ufrj.br).

Data analysis and construction of databases

Data originated in the *T. cruzi* genome project are available from the WWW server at the Department for Biochemistry and Molecular Biology (Wim Degrave), IOC/Fiocruz at http://www.dbbm.fiocruz.br/genome/tcruzi/tcruzi.html. It contains biological and parasitological data on CL Brener, its karyotype, all available *T. cruzi* sequences from GenBank, organized in classes, data on the EST-sequencing project and on available libraries, a *T. cruzi* codon table, constructed from housekeeping gene sequences, and also a listing of activities and participating groups in the *T. cruzi* Genome Initiative. A first version of TcruziDB is being constructed in the ACeDB software 4.3. Moreover, a *T. cruzi* discussion list has been started (subscription requests to majordomo@iris.dbbm.fiocruz.br; messages to tcruzi-1@iris.dbbm.fiocruz.br), reaching more than 150 subscribers from 26 countries.

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Programmed Cell Death in Trypanosomatids

S.C. Welburn, M.A. Barcinski and G.T. Williams

It has generally been assumed that apoptosis and other forms of programmed cell death evolved to regulate growth and development in multicellular organisms. However, recent work has shown that some parasitic protozoa have evolved a cell suicide pathway analogous to the process described as apoptosis in metazoa. In this review, Susan Welburn, Marcello Barcinski and Gwyn Williams discuss the possible implications of a cell suicide pathway in the vector-borne Trypanosomatids.

It is a tenet of current thinking in cell biology that programmed cell death (PCD) evolved to meet the particular needs of multicellular life[1,2]. This idea is not based on experimental evidence showing that all unicellular organisms lack PCD, but rather is a reflection of the current excitement about the now obvious benefits of PCD for multicellular life forms in particular. When we consider the problems involved in, for example, mammalian differentiation or immune defence, some sort of control system on cell number and a harmless disposal mechanism for unwanted cells appears to be essential. As a result of extensive investigation of cell death pathways in metazoa, apoptosis is now widely accepted as a universal component of developmental and differentiation programmes and as a defining step in oncogenetic pathways of mammalian organisms. Active cell death is important in multicellular organisms to maintain stable cell numbers, and it fulfills specialist selection and control functions in many systems such as the immune, haemopoietic and nervous systems. The failure of such cell control systems in mammals may lead to cancerous growths and, within the immune system, to the development of autoimmune disease[3]. It is generally assumed that a regularized system of cell death, among other essential development programmes, appeared in phylogenesis after the onset of multicellularity.

Programmed cell death is clearly not confined to mammals and is extremely widespread, possibly universal, in multicellular animals. Much of the genetic analysis of PCD has been carried out on the nematode *Caenorhabditis elegans*[4] and, more recently, on the fruit fly *Drosophila*[5]. Implicit in the specific association of PCD with multicellularity is the idea that the protozoa would have no need of an orderly process of cell death; what would be the point in a single-celled organism carrying the complex machinery for arranging its own death? How could there be any genetic advantage in PCD for such single-celled organisms? This leads inevitably to the idea that, unless killed by outside forces (inducing 'necrotic death'), protozoa have achieved a state of immortality with no apparent limits to their proliferation. Acceptance of this line of thought permeates much of parasitology. However, the boundaries defining regulated cell death pathways as a

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