Characterisation of a novel interspersed *Toxoplasma gondii* DNA repeat with potential uses for PCR diagnosis and PCR-RFLP analysis

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**Abstract**

A novel *Toxoplasma gondii* interspersed repeat element (TgIRE), present in most of the tachyzoite chromosomes, was characterised. Two regions on the TgIRE sequence showed high identity to two different *T. gondii* expressed sequence tag cDNAs of unknown function, which seems to be TgIRE pseudogenes. Two set of primers were designed, 2-2 and 2-3, that amplify products of 1.02 and 0.62 kb, respectively. *T. gondii* DNA from RH and Me49 strains was amplified with TgIRE 2-2’ primers, and the respective 1.02 kb products were digested with several endonucleases. Different fragment patterns by gel electrophoresis were found only with *MboI*. Sensitivity analysis revealed that the set 2-3 was more sensitive than 2-2’, detecting by gel visualisation the amount of DNA equivalent to 1 and 10 parasites, respectively.

**Keywords:** Interspersed DNA repeat; Polymerase chain reaction; Restriction fragment length polymorphism; Pseudogene; *Toxoplasma gondii*

1. Introduction

*Toxoplasma gondii* is an obligate intracellular parasite responsible of widespread infections among mammals and birds. In humans, *T. gondii* is known to cause transplacental infections that can lead to abortion or to severe neonatal malformations, and recently as an opportunistic pathogen of major importance in immunocompromised individuals.

Despite a substantial part of the eukaryote genome consists of repetitive DNA sequences, only two families of such elements have been described in *T. gondii*. One of them is an interspersed DNA repeat that contains mitochondrial-like sequences flanked at both ends by 91-bp direct or inverted repeats [1]. The other is a tandemly repeated element called ABGTg7 or TGR [2,3] located near the telomeres as well as in other parts of the largest chromosomes [3].

Repeated DNA elements could be useful as tools for diagnosis as well as for restriction fragment length polymorphism (RFLP) analysis. Regarding diagnosis, active *T. gondii* infection could be detected from clinical samples using ABGTg7 as a probe [4]. Thus, primers designed from the TGR1E sequence are commonly used for polymerase chain reaction (PCR) for the diagnosis of toxoplasmosis [5,6]. Genome heterogeneity among parasite strains was evidenced using the repeated TGR1E element as a hybridising probe but RFLP analysis did not correlate with virulence [7]. Literák et al. [8] analysed other *T. gondii* strains for their TGR1E-RFLPs, finding two defined groups: virulent (n = 3) and avirulent (n = 19) strains. Random amplified polymorphic DNA analysis also revealed the same two groups [9].

In this study, we describe a novel family of a repeated DNA element, *T. gondii* interspersed repeat element (TgIRE), that may have uses in the diagnosis of toxoplasmosis and PCR-RFLP analysis.
2. Materials and methods

2.1. Screening

A cosmid genomic library of RH strain (AIDS research and Reference Reagent Program) was hybridised in situ with a $^{32}$P-20-mer oligonucleotide (5'-AGGCTTTAGGGTTAGGGTT-3'). Hybridisation and washes were performed in 6×SSC (1×SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7) plus 0.25% non-fat dry milk at 50°C.

2.2. Sequence analysis

DNA sequencing was performed using a Perkin Elmer ABI 377 machine and a BigDye Terminator Cycle Sequencing kit (Perkin-Elmer). Internal oligonucleotides 1', 2', 2' and 3 were designed (see PCR analysis). Sequence similarity searches were performed using the Blast program of the National Center for Biotechnology Information (NCBI) web page. The TgIRE accession number (AN) is AF177728.

2.3. Genomic and pulse field gel electrophoresis (PFGE) Southern blot analysis

Genomic DNA and chromosomal DNA agarose blocks were prepared as described previously [3,4]. Following electrophoresis, gels were blotted onto nylon membranes (Hybond, Amersham) by vacuum (Pharmacia LKB, VacuGene XL). Hybridisation was performed in 6×SSC plus 0.25% non-fat dry milk at 65°C, and washing at high stringency in 0.1×SSC, 0.1% SDS at 65°C.

2.4. PCR and RFLP analysis

Amplifications were performed in a Perkin Elmer thermal cycler (GeneAmp 9600) with 2.5 U of Taq DNA polymerase (BRL) in a final volume of 50 μl with 1×BRL buffer plus 2.5 mM MgCl₂, 0.1 mM (each) deoxynucleotide triphosphate and 1 μM of each oligonucleotide. The following TgIRE (5'–3') oligonucleotides were designed: 1, TACCCTTTTCCGATGCCGTC; 1', GCATTTGGCTATATGATCTAC; 2, ACCTGTTGTCACCCTCTCTG; 2', TGCACTGGCATATTTAATGG and 3, ACAGCTGTGTAAAGCATTTG; as well as B1 primers [10]. Samples were amplified for 35 cycles as follows: 1 min at 94°C, 40 s at 52°C and 1 min 30 s at 72°C. An initial step of 5 min at 94°C and a final step of 10 min at 72°C were included.

3. Results and discussion

3.1. Cloning of the T. gondii TgIRE element and analysis of its genomic organisation and sequence

A cosmid T. gondii genomic library was screened using a $^{32}$P-20-mer probe based on an Eimeria tenella telomeric-like sequence. This sequence, with internal T₆/G₆ repeats, is present in all E. tenella chromosomes but was not sensitive to Bal31 digestion, suggesting a non-telomeric array for this element [11]. From an initial screening, five clones were chosen for analysis. By Southern blot the 20-mer probe hybridised strongly only to a 1.9-kb EcoRI fragment (called TgIRE) of the recombinant cosmid 2 (data not shown).

Subsequently, the TgIRE fragment was used as a $^{32}$P-hybridisation probe against restriction endonuclease digested T. gondii DNA and PFGE separated chromosomes transferred to nylon membranes (Fig. 1). Southern blot analysis of genomic DNA revealed that TgIRE is an interspersed repeat element (Fig. 1A). Chromosomal distribution of TgIRE analysed by PFGE Southern blot showed once again that TgIRE is interspersed along the genome, hybridising with different intensity to the most of parasite chromosomes (Fig. 1B).

The nucleotide sequence shows that TgIRE is a 1919-bp element that does not show an obvious telomeric-like repeat. There is a region (from nucleotide 433 to 452) which has 80% identity with the 20-mer probe and there are several stretches of multiple A, T and C nucleotides along

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Fig. 1. Genomic organisation of TgIRE repeated element. A: Autoradiography of a Southern blot of genomic T. gondii RH strain DNA (RH gDNA) digested with EcoRI (E), SalI (S), BamHI (B) and AccI (A) and recombinant cosmid 2 (C2) digested with EcoRI, and hybridised with $^{32}$P-labeled TgIRE probe. Cosmid 2 revealed the expected 1.9-kb EcoRI fragment. λDNA-HindIII restriction fragments were used for size calibration (right). B: T. gondii RH strain chromosomes were separated by PFGE (RH Ch-PFGE), stained with ethidium bromide (EtBr), blotted onto nylon membrane and hybridised with $^{32}$P-labelled TgIRE. Roman numeral (left) identifies individual chromosomes. The 1.9-kb TgIRE EcoRI fragment was cloned into pBluescript (pTgIRE). In order to obtain TgIRE probe, pTgIRE was digested with EcoRI enzyme and the fragment was purified from agarose gel by a DNA purification kit (Quagen) and $^{32}$P-labelled by random priming.
the TgIRE sequence. We propose that the 20-mer probe could have hybridised either to this similar region or to A and C stretches. Several direct (DR) and inverted (IR) repeats were found along the TgIRE element, being the longest DR of 20/21 bp (Fig. 2A). The TgIRE sequence did not reveal strong identity with any defined gene by homology searches. However, searching on the Toxo-
plasma Expresssed Sequence Tag (EST) database (http://
www.cbil.upenn.edu/ParaDbs/Toxoplasma/index.html) displayed high identity of two regions on TgIRE (-A and -B) with two uncharacterised T. gondii cDNAs: zy87d12 (AN: W06019) and zy60e03 (AN: N61085), respectively (Fig. 2B). TgIRE-A and -B, and cDNA versions, do not have long ORFs inferring that at least TgIRE-A and -B are unable to express parasite proteins. Moreover, whereas one part of the TgIRE-B aligns with the first 138 nucleo-
tide of cDNA (zy06e03) as reverse complementary se-
quence, the other part aligns directly (Fig. 2B). It therefore seems that TgIRE-B inverts on itself. This data suggest that these TgIRE cDNA-like regions could be pseudo-
genesis, raising the question as to the origin of the genes.

Amino acid searches of TgIRE pseudogenes revealed some
identity to transcription factor GATA [12]; integrin β-8 precursor, a family of molecules involved in cell-cell and cell-matrix adhesion [13]; and Saccharomyces cerevisiae ESR, a multifunctional protein which monitors completion of DNA repair synthesis [14] (Fig. 2C). However, the data are insufficient to draw a reliable conclusion.

3.2. PCR amplification and RFLP analysis

RH and Me49 parasite DNA, and pTgIRE plasmid were amplified using TgIRE 1-1’, 2-2’ and 2-3 primers. This allowed the detection of the respective fragments: 1.55 kb, 1.02 kb and 0.62 kb (data not shown). This data infer that the TgIRE element is also contained in Me49 strain. In order to investigate polymorphism between RH (virulent) and Me49 (avirulent) strains, the respective 1.02-kb fragments (primers 2-2’) were digested with several endonucleases. Enzymes AluI, HpaI, and AccI did not reveal any differences in fragment patterns among PCR fragments obtained either from RH or Me49 DNA source (data not shown). This suggests that TgIRE elements are highly conserved between RH and Me49 T. gondii strains. However, variation in fragment pattern resulting from site gain or loss was observed by MboI digestion (Fig. 3A). Based on the MboI findings, further studies are being considered to analyse the value of this TgIRE-PCR-RFLP analysis in clinical samples.

The sensitivity of the PCR amplification developed with TgIRE primers 2-2’ and 2-3 was also studied. As reference, B1 primers were used [10]. Using dilutions of DNA from purified RH tachyzoites, PCR amplification allowed the detection of 0.1 pg of DNA, equivalent to 1 tachyzoite, when primers 2-3 were used. This was compared with the sensitivity of primers B1 of 1 pg (Fig. 3B) as well as with primers TgIRE 2-2’ (data not shown). Recently, Pelloux et al. [15] designed a new set of B1 primers which can detect DNA equivalent to 1 tachyzoite by direct gel visualisation. We consider that TgIRE 2-3 primers should be included into the group of the most sensitive set of primers for T. gondii PCR amplification with the new B1 set [15] and those derived from ribosomal DNA and TgRIE sequences [6]. As a 0.62-kb fragment is amplified with TgIRE 2-3 primers, they could be used in association with those derived from TgRIE, ribosomal DNA or B1 sequences for the rapid and sensitive diagnosis of T. gondii infection.

References


ACKNOWLEDGMENTS

S.O.A. (researcher) and V.M. (fellow) are members of the National Research Council (CONICET). This work was supported by Grants from The Third World Academy of Sciences, Trieste, Italy (RG/BIO/LA 96-337).


