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Isolation of the Plastid DNA of *Toxoplasma gondii*

SIAN C. MUNRO AND ALAN M. JOHNSON

Molecular Parasitology Unit, Department of Cell and Molecular Biology, University of Technology, Sydney. Westbourne St. Leonards, NSW 2065, Australia

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ABSTRACT

The plastid DNA from different strains of *Toxoplasma gondii* has been isolated using a variety of techniques. The relative effectiveness of these isolation techniques is compared. Initially, clones were isolated from genomic DNA libraries, but 100% positive identification of these clones proved difficult, due to variations in the plastid sequences and the lack of comparable sequences. Purification techniques using commercial columns gave eluates highly enriched in plastid DNA, but other contaminating DNA was also shown to be present. Finally, the novel long polymerase chain reaction technique (long-PCR) was trialed and was found to be the most efficient isolation and purification method.

INTRODUCTION

Toxoplasma gondii is an obligate intracellular protozoan parasite which belongs to the phylum Apicomplexa. It is the causative agent of toxoplasmosis which is widespread in humans and other vertebrates (Dubey 1986; Frenkel 1988). Toxoplasmosis is usually asymptomatic in healthy, immunocompetent people. However, immunocompromised individuals are very susceptible to the parasite and infection may cause major organ damage, encephalitis or even death (Frenkel et al. 1975; Luft et al. 1984; Luft and Remington 1992).

Recent research into Apicomplexan parasites has lead to the discovery of two extrachromosomal pieces of DNA. One is a 6 kb linear strand of DNA which has been identified as the mitochondrial genome (Vaidya et al. 1989), and the

other is a 35 kb circular genome which has characteristics similar to the plastid DNA of plants and algae (Wilson et al. 1996). The presence of the plastid DNA within these parasites is particularly interesting as it is the first plastid genome to be identified outside the plant kingdom. Within plant and algal cells, plastid DNA is known to be involved in gene expression and photosynthesis. However, its function in Apicomplexan cells has yet to be elucidated. The Apicomplexan plastid genome has been shown to be transcriptionally active and hence, it is thought to play an important, if not essential, role in the cellular mechanisms of these parasites (reviewed in Feagin 1994; Jeffries and Johnson 1996).

It is anticipated that the plastid DNA present in *T. gondii* will be a useful tool for investigating the genetic diversity of this parasite and for clarification of the complex phylogenetic relationships seen among the Apicomplexa (reviewed in Johnson 1997). Characterisation of the Apicomplexan plastid DNA may also lead to the identification of novel drug targets within these parasites, as the plastid DNA is thought to have evolved from an ancient endosymbiont whose closest living relatives are the cyanobacteria. The plastid genome therefore, has a different ancestral origin to mammalian DNA and drugs may be targeted to exploit such differences. Most of the work conducted on the plastid DNA in Apicomplexans has focused on *Plasmodium* sp. (Williamson et al. 1985; Wilson et al. 1996; Yap et al. 1997). The purpose of this study was to use the plastid isolation techniques from *Plasmodium* studies to isolate the plastid DNA from different strains of *T. gondii*.

MATERIALS AND METHODS

Parasite cultures

The three strains of *T. gondii* used in this study were grown as tachyzoites in cell culture. The RH and Me49 strains were grown in a mouse macrophage cell line (RAW 264.7) whilst the S48 strain was grown in a primate fibroblast cell line (Vero). The cells were cultured in RPMI 1640 supplemented with 2% foetal bovine serum, penicillin G (50 μg/ml) and streptomycin (50 μg/ml). Flasks were incubated at 37°C in a 5% CO_2/95% air atmosphere.

Genomic DNA extraction and purification

The genomic DNA was extracted from the tachyzoites of the strains of *T. gondii* using a phenol/chloroform extraction procedure as described in Johnson et al. (1986) with the omission of the sodium perchlorate step.

Construction of the λGEM11 genomic DNA libraries

Genomic DNA was packaged into the λGEM11 vector according to the

manufacture's specifications (Promega-λGEM11 technical bulletin). Briefly, the genomic DNA was partially digested using *Sau*3A1 and cloned into λGEM11 with partially filled *Xho*1 arms. These ligations were then added to Promega Packagene extracts.

Polymerase Chain Reaction (PCR)

A fragment of the large subunit ribosomal RNA gene (lsu rRNA) was amplified using primers designed from the aligned plastid DNA sequences of *T. gondii* (Beckers et al. 1995; *T. gondii* plastid sequence accession no. U18086) and *P. falciparum* plastid DNA (accession no. X61660). The elongation factor Tu (*Tuf*A) gene was also amplified using primers described elsewhere (Gleeson and Johnson, in press) (Table 1).

The PCR reactions were conducted in a 100 μl solution which contained 25 mM dNTPs, 25 mM $MgCl_2$, 50 pmol primers, 1 unit Taq, 1×Taq buffer and 50-100 ng of template DNA. Each reaction was carried out in a 0.5 ml microtube and overlaid with 100 μl of mineral oil. The amplification programs consisted of 30 cycles starting with a 2 min denaturing step at 92°C, followed by 2 min of primer annealing at temperatures ranging from 48-55°C and finishing with 2 min of primer extension at 72°C. The PCR products were separated by agarose gel electrophoresis and visualised by ethidium bromide staining.

Radioactive probing of the genomic DNA libraries

Probes were labeled with radioactive ^{32}[P]-dATP in the presence of Tris-HCl pH 7.5. $MgCl_2$, dNTPs, random nanomers and 1 unit of Klenow fragment. The reaction was incubated for at least 20 min at 37°c and terminated by the addition of EDTA (20 mM final conc). Immediately before use in a hybridisation experiment the mixture was denatured by heating at 95°C for 5 min and snap chilled on ice.

Isolation of positive clones

The transfer of plaques to nitrocellulose filters and the subsequent isolation of positive plaques was accomplished following the manufacturer's instructions (Promega-λGEM11 technical bulletin).

Hybridisation

Nitrocellulose membrane filters were prehybridised for at least 2 hrs at 65°C in 263 mM Na_2HPO_4 pH 7.2, 1 mM EDTA, 7% SDS, and 1% BSA. The prehybridisation mix was then discarded and another 50 ml of hybridisation solution plus the radioactively labeled probe was added and left to hybridise

overnight. After hybridisation the probe mix was removed and the membranes were washed twice with 2×SSC (30 mM $Na_3C_6H_5O_7$ and 300 mM NaCl, pH 7.0) and 0.1% SDS for 30 min at 65°C, followed by two washes in 0.1% SSC and 0.1% SDS for 30 min at 65°C.

DNA purification

Phage lysate was prepared following the manufacturers instructions (Promega-λGEM11 technical bulletin). DNAse (1 μg/ml) and RNAse (10 μg/ml) were then added and the mixture was incubated with shaking for 30 min. 1 M NaCl and 10% PEG-6000 were dissolved into the lysate and the mixture was left at 4°C overnight. The phage-PEG complex was pelleted by centrifugation at 10,000 rpm for 15 min at 4°C. The supernatant was discarded and the pellet was resuspended with 500 ml of TMN buffer (10 mM Tris-HCl pH 7.5, 10 mM Mg^{2+} and 100 mM NaCl). The solution was then transferred to a 1.5 ml microtube and extracted twice with an equal volume of chloroform, retaining the aqueous layer. An equal volume of saturated aqueous phenol was added, the solution was mixed by inversion and spun for 2 min at maximum speed in a microfuge. The aqueous layer was retained and the extraction process was repeated using aqueous phenol:chloroform (1:1) and again using chloroform:isoamyl alcohol (24:1). The phage DNA was precipitated out by adding 50 μl of 3 M sodium acetate and 1.5 ml of ethanol and leaving the solution for at least 1 hr at -20°C. The DNA was pelleted by centrifugation in a microfuge at maximum speed for 10 min. The pellet was washed with 70% DNA grade ethanol, vortexed briefly and centrifuged again. The pellet was then dried in a desiccator for 2 hrs and resuspended in 100 μl of buffer TE (10 mM Tris-HCl pH 8.0 and 1 mM EDTA). The DNA was quantitated and assessed for purity using a GeneQuant spectrophotometer.

DNA sequencing

Direct sequencing of the plaque DNA was accomplished using a modified version of dideoxy chain termination sequencing (Sanger et al. 1977). The 17 μl reactions were carried out using a SequaTherm™ LongRead™ cycle sequencing kit and contained 2 μg of template DNA, 5 pmol of T7 Promoter or SP6 Promoter dye-labeled primer (Li-Cor), 1×SequaTherm™ buffer and 1.5 units of SequaTherm™ DNA polymerase. 4 μl aliquots of this mix were added to 0.5 ml microtubes each of which contained 2 μl of one of the termination dNTPs. The amplifilcation program consisted of 35 cycles which began with a 30 sec denaturation at 92°C, followed by 10 sec annealing at 40°C and 30 sec annealing at 50°C, finishing with 30 sec of extension at 70°C. The reactions were conducted without oil on a MJ PTC-100 Thermal Cycler with a heated lid function. Each

reaction was terminated by the addition of 4 ml of stop solution/running dye (95% v/v formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue). The four sequencing reactions (G, A, T and C) for each sample were loaded side by side on a Li-Cor 66 cm (0.25 mm thick) polyacrylamide gel preheated to 45°C. The gel was run at 2,000 V, 25 mA and 45 W for at least 12 hrs. An image of each run was stored on an IBM compatible computer using the BaseImagIR v2.30 software package which was also used in the reading of the sequences.

Sequence analysis

The sequence obtained from the automated sequencer was analysed and compared with other sequences available on GenBank using the Blastn program available through the Australian Genomic Information Service Network (ANGIS).

Ultracentrifugation

Method A: 100 μg of DNA was pipetted into an ultracentrifugation tube covered in foil. 1 ml of Hoechst dye (100 μg/ml) was added along with 200 μl of 10×*Eco*R1 buffer and H_2O to make up a final volume of 3 ml. The mixture was then left for 10 min before the addition of 7.2 ml of saturated cesium chloride (CsCl). The air remaining in the tube was removed by the addition of mineral oil before the tube was sealed. The tube was then spun for 22 hrs at 45,000 rpm, after which time the DNA was viewed using a UV light and extracted from the tube using an 18G needle and syringe. The Hoechst dye was removed by extraction with an equal volume of isopropanol. After mixing by inversion the solution was centrifuged for 5 min at 4°C. This extraction was repeated six times. The CsCl was removed by diluting the DNA solution with H_2O (3:1) and ethanol (2:1, v: volume of undiluted DNA). The solution was gently mixed by inversion and left at 4°C for 15 min. The DNA was pelleted by centrifugation for 15 min at 4°C, washed with 70% ethanol, air dried and finally resuspended in TE buffer.

Method B: As outlined in Cardner et al. (1988).

Qiagen Tip-20 DNA purification

The plastid DNA was extracted using Qiagen Tip-20s following the Qiagen protocol. The amounts of P1, P2 and P3 buffers added in the first three steps of the method were increased from 0.3 ml to 0.5 ml. The DNA was run on a 0.6% agarose gel and visualised by ethidium bromide staining.

Southern blotting

DNA from agarose gels was transferred to nitrocellulose membranes for probing using the method described in Sambrook et al. (1989).

Long PCR

The long PCR reactions were conducted according to the manufacturer's instructions (Expand^{TM} 20kb^{Plus} PCR System) with the following modifications: dNTPs were added in a final concentration of 2 mM, the amount of template DNA added was increased to 1 μg and the amount of Expand^{TM} 20 kb^{Plus} enzyme mix was reduced to 0.5 μl per reaction. The reactions were run on an MJ Research PTC-200 DNA Engine for 25 cycles with a 10 sec denaturing step at 92°C, 30 sec annealing at 55-60°C and 12 min extension at 68°C. The products were run on a 0.6% agarose gel to separate the bands which were visualised by ethidium bromide under UV light.

Table 1. Primer Designs

Gene	Primer	Reference
lsu rRNA	fwd: 5' TACAGACTTTTCACATCAGG 3'	(this study)
lsu rRNA	rvs: 5' TTTACATCGGATACGGACCG 3'	(this study)
Tuf A	fwd: 5' GCTCATATTGATTGTCCTGG 3'	(Gleeson and Johnson, in press)
Tuf A	rvs1: 5' CCTGCTCCTATAGT 3'	(Gleeson and Johnson, in press)
Tuf A	rvs2: 5' CCAGGACAATCAATATGAGC 3'	(Gleeson and Johnson, in press)
rpo C	fwd1: 5' CCCATTTGATCTCCATCAAAATCAGC 3'	(this study)
rpo C	fwd2: 5' TCGTTTCTTCTTAATAGTGGTGATAAAT 3'	(Fichera and Roos 1997)
rpo C	rvs: 5' AGCTACTCCTATTGCCCATAAATGGT 3'	(Fichera and Roos 1997)
ssu rRNA	rvs: 5' TACGGCTACCTTGTTACGACTTCA 3'	(Egea and Lang-Unnasch 1995)

RESULTS

PCR analysis

To determine whether the plastid DNA was present in all of the strains of *T. gondii*, an initial PCR was conducted using primers designed to a section of the lsu rRNA gene. All of the *T. gondii* strains available in our laboratory were included in this study as template DNA for the PCR reactions. A 594 bp product was seen in all eight reactions which had virulent strains as template DNA and in all seven reactions which had avirulent strains as template DNA (data not shown).

Probing of genomic DNA libraries

Lambda libraries were constructed using the genomic DNA from three different strains of *T. gondii:* the virulent strain RH, the avirulent strain Me49 and the mutant strain S48. Each library was probed with radioactive ^{32}P-labeled PCR products obtained from the lsu rRNA and the *Tuf*A genes on the plastid genome. Using the lsu rRNA probe, nine positive clones were isolated from the RH library, eight from the Me49 library and five from the S48 library. Similarly, using the *Tuf*A probe, five positive clones were isolated from the RH library, two from the Me49 library and eight from the S48 library. DNA from the clones was obtained and sequenced directly from the 5' end using a T7 Promoter dye-labeled primer, and from the 3' end using a SP6 Promoter dye-labeled primer. Initial attempts at sequencing the DNA were not successful. However, optimisation of the PCR reaction, the use of high quality *Taq* polymerase and the very quick ramping times of a new MJ cycler 100 PCR machine gave very clear sequencing runs up to 900 bp in length.

Sequence analysis

The resulting sequences were submitted to the Blastn search program in ANGIS for identification. Of the clones isolated with the lsu rRNA probe, one RH, three Me49 and one S48 clone were positively identified as *T. gondii* plastid DNA. These sequences showed 96-98% homology when compared with the entire *T. gondii* plastid genome which had previously been submitted to GenBank (accession no. U18086). However, only one end of each clone aligned with a section of the *T. gondii* plastid. It was not possible to sequence the RH clone using the T7 primer. The three Me49 clones all showed homology (67-73%) to human DNA when sequenced from the 3' end, whilst the S48 clone was highly homologous to *E. coli* DNA when sequenced from the 3' end. None of the clones isolated using the *Tuf*A probe were identified as *T. gondii* plastid DNA.

Ultracentrifugation

DNA from both the RH and the Me49 strains was purified by ultracetrifugation using two different CsCl gradient fractionation methods. The first method used Hoechst dye to visualise the DNA, whilst the second used ethidium bromide. Both methods used differing amounts of CsCl and different spin times. In both cases only the genomic DNA band was apparent, with no evidence of a satellite, circular DNA band.

Column purification

Isolation of the entire plastid DNA from the three strains of *T. gondii* was

also attempted using Qiagen Tip-20s. When visualised on a gel, the purified DNA solution ran as a smear with a single band visible at approximately 30-35 kb (Fig. 1). Southern blots of gels containing both the purified plastid DNA and genomic DNA were probed with both radioactive ^{32}P-labeled *Tuf*A and lsu rRNA plastid probes. In both cases more probe hybridised to the purified plastid DNA than to the genomic DNA. The blot probed with the lsu rRNA probe was also considerably more intense than that probed with the *Tuf*A. Multiple banding was seen in the lane containing the plastid DNA with both probes, although more bands were highlighted by the lsu rRNA probe. Minimal or no binding was seen in the lanes containing the Vero and RAW cell DNA lanes.

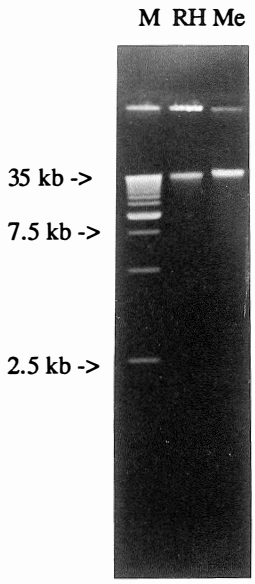

Figure 1. Column purified plastid DNA. Lanes 1: 2.5 kb Molecular Ruler marker (BioRad), 2: Purified RH plastid, 3: Purified Me49 plastid.

Long PCR

Finally, long PCR was trialed for isolation of the plastid DNA. Primers were designed for regions on the plastid genome that gave end products which were 10.6 kb, 11.0 kb or 11.6 kb and 13.7 kb in length (Table 1, Fig. 2). Bands were obtained from the RH and Me49 strains of *T. gondii,* as visualised by ethidium bromide on an agarose gel. Positive identification of these PCR products was obtained by southern blots and DNA sequencing.

DISCUSSION

Within the Apicomplexa, plastid DNA has been identified in species from the three major groups of Apicomplexans: the haemosporins - *Plasmodium* sp.,

(Wilson et al. 1996; Yap et al. 1997) the coccidia - *Toxoplasma, Neospora* and *Eimeria,* (Borst et al. 1984; Egea and Lang-Unnasch 1995; Gleeson and Johnson in press; Köhler et al. 1997) and the piroplasms - *Babesia* and *Theireria* (Gozar and Bagnara 1995; Wilson et al. 1996). The presence of the plastid DNA in these major groups implies that it may be found across a wide range, if not in all Apicomplexans. It was not surprising then, that the initial PCR conducted in this study showed that the plastid DNA is present in all of the strains of *T. gondii* available in our laboratory. The strains used in the experiment were a representative sample of *T. gondii* strains and on this basis it is postulated that the plastid DNA would be present in all strains of *T. gondii.*

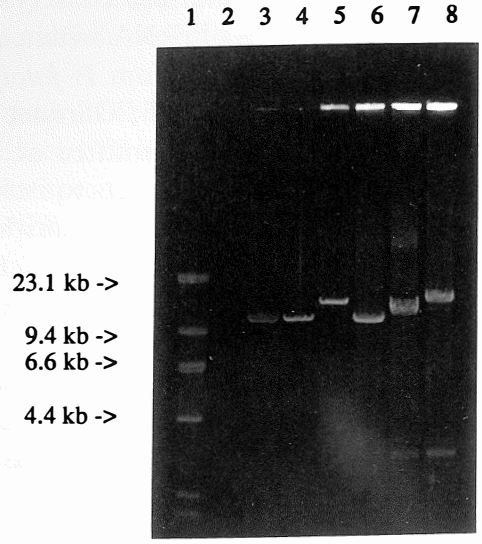

Figure 2. Long PCR products. Lane 1: Lambda *Hind*III marker; lane 2: negative control; lane 3-5: Me49 10.6 kb, 11.0 kb and 13.7 kb PCR fragments; lanes 6-8: RH 10.6 kb, 11.6 kb and 13.7 kb PCR fragments.

Probing of genomic DNA libraries led to the isolation of multiple positive clones from each of the strains of *T. gondii.* The libraries were probed with both the lsu rRNA and *Tuf*A plastid gene fragments, as these two genes are located opposite one another on the gene map of *P. falciparum* (Wilson et al. 1996). Hence it was anticipated that the clones isolated from the libraries using these probes would encompass the entire 35 kb plastid genome. Unfortunately, only the sequence from one end of each of these clones aligned with the available plastid sequences in GenBank, which meant that 100% positive identification of these clones was not possible. There are variations in the sequence of the plastid DNA among the different strains of *T. gondii* (S. Munro, unpublished data). Also, multiple copies of the plastid genome have been reported to be present in each tachyzoite and their sequences may vary (Fichera and Roos 1997; Köhler et al.

1997). Such variations, plus the fact that only one *T. gondii* plastid sequence is available in GenBank (compared with the multitude of human and *E. coli* sequences present) means that the likelihood of a variable section of our *T. gondii* plastid sequence aligning to the sequence present in GenBank is minimal.

In all of the isolated positive clones, the ends which were identified as *T. gondii* plastid aligned with sections of the plastid genome which contain the large and small subunits of the rRNA genes. The sequence alignments showed a high homology (96-98%) which indicates that the plastid rRNA genes are highly conserved. Such a result is supported by the initial PCR, which showed that the amplified fragments of the lsu rRNA gene were of similar size in all of the *T. gondii* strains. Similarly, nuclear rRNA genes have previously been shown to be conserved among strains of *T. gondii* (Luton et al. 1995) and such conservation is consistent with the relatively specific mechanical functions of rRNA within cells.

Extrachromosomal DNA has previously been isolated from *P. falciparum* by means of CsCl ultracentrifugation (Williamson et al. 1985; Gardner et al. 1988). In the latter of these studies the isolated DNA was identified as plastid based on electron microscopy results and DNA sequencing. Attempts to isolate the plastid DNA from *T. gondii* tachyzoites using two different ultracentrifugation techniques showed only the presence of the nuclear DNA. As the *P. falciparum* plastid genome is known to be very A/T rich (Williamson et al. 1985) it would have a different gradient buoyancy to that of *T. gondii* and hence the *T. gondii* plastid genome may not have visibly separated from the nuclear genome under the conditions used. Also, the number of parasites used in the experiments may need to be increased as a high concentration of DNA is required to visualise the plastid band (Gardner et al. 1988).

Isolation of the plastid DNA via commercially available columns has been previously reported for the murine malarial species *Plasmodium berghei* (Yap et al. 1997). The results of the current study showed that when *T. gondii* genomic DNA is applied to the column, the eluate is highly enriched in plastid DNA. In PCR experiments, DNA fragments from the lsu rRNA were readily obtained when the eluate was used as template. When viewed on an agarose gel, distinct bands are present at approximately 35 kb (Fig. 1), and these bands were highlighted in southern blots probed with radioactively labeled fragments of the plastid lsu rRNA and *Tuf*A genes. A number of other bands were also highlighted by the different plastid probes. The lsu rRNA probe highlighted more bands than the *Tuf*A probe and it highlighted those bands more intensely. Such a result can be explained by the fact that there are two copies of the rRNA genes (arranged in a characteristic inverted repeat) on the plastid genome, and hence more probe is bound. The multiple bands present in the gel are probably the result of the nicked

and supercoiled circular plastid molecules running at different positions on the agarose gel than the relaxed circular, plastid counterpart. The presence of bands larger than the 35 kb plastid may also be due to linearised fragments of plastid DNA recombining into dimers and trimers. Similar multiple banding has been described in pulsed field DNA studies conducted with *Eimeria tenella* (Smith and Cantor 1987), *T. gondii* (Williamson et al. 1999) and *Neospora caninum* (Gleeson and Johnson, in press).

The column eluate also contained other smaller pieces of DNA which appeared as a smear and which did not bind to the plastid lsu rRNA and *Tuf*A probes or to a nuclear rRNA probe. These smaller fragments of DNA appear to be linear when viewed by electron microscopy (data not shown). It is hypothesised that many of these linear pieces of DNA are the 6 kb mitochondrial element of the parasite, which are often arranged in head-to-tail oligomers (Vaidya and Arasu 1987).

Long PCR is a novel technique which can be used to synthesise products which exceed 20 kb in length. In the current study, long PCR was effectively used to isolate specific fragments of the plastid DNA. PCR primers were designed to obtain PCR products which covered almost the entire 35 kb plastid genome. The products were approximately 10.6 kb, 11.0 kb or 11.6 kb and 13.7 kb in length and were isolated from both the RH and the Me49 strains of *T. gondii* (Fig. 2). Careful selection of primer regions and high quality template DNA were found to be the most important components of a successful reaction. Both column purified genomic DNA and column purified plastid DNA were found to be unsuitable to use as template. The column purification process presumably contains components which inhibit the long PCR reaction. Southern blots, probed with the two plastid probes, confirmed that both the 11.0 or 11.6 kb and the 13.7 kb bands contained sections of the lsu rRNA gene whilst the 10.6 kb band contained a section of the *Tuf*A gene. The PCR products will now be used in the construction of restriction enzyme maps to identify polymorphisms in the plastid DNA among the different strains of *T. gondii*.

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