MATERIALS AND METHODS
2. **Materials and Methods**

2.1. **Materials**

Cell culture media, culture flasks and culture dishes, chemicals, restriction enzymes, PCR enzymes, DNA purification kits, X-ray films, membranes and radioisotopes were obtained from the following companies:

**Sigma**- RPMI-1640 media, sodium bicarbonate, glucose, gentamycin, D-sorbitol, saponin, BND-cellulose, sucrose, guanidium thiocyanate, ammonium acetate and PCR primers, ammonium per-sulfate, TEMED, bis-acrylamide, calcium chloride, hypoxanthine, ethidium bromide, xylene cyanol, bromophenol blue, salmon sperm DNA

**GIBCO-BRL (Invitrogen)**- Platinum Taq DNA polymerase, Platinum px DNA polymerase, 100bp DNA ladder, 1kb DNA ladder, DNase, dATP, dGTP, dTTP, dCTP, Albumax II, Superscript™ first strand synthesis system for RT-PCR, Superscript™ one step RT-PCR kit

**New England Biolabs**- Restriction enzymes and buffers, T4 DNA ligase, calf intestinal alkaline phosphatase, T4 polynucleotidyl kinase

**Promega**- Taq DNA polymerase with buffer, restriction enzymes and buffers, T4 DNA ligase

**Amersham**- Lysozyme, Tris-saturated phenol, proteinase K, T4 polynucleotidyl kinase, agarose, X-ray film (Hyperfilm-MP), Hybond N+

**Tarsons**- 25cm² culture flasks, 75cm² culture flasks, 60 mm culture dishes, 90 mm culture dishes, disposable sterile pipettes

**SRL**- Sodium dodecyl sulphate, Tris base, sodium acetate, sodium chloride, isoamyl alcohol, sodium hydroxide, acrylamide

**Ranbaxy**- EDTA, chloroform

**Merck**- Isopropanol

**Qiagen**- Qiagen plasmid mini kit, Qiagen plasmid midi kit

**Jonaki, India**- [γ-³²P]-ATP, [α-³²P]dATP

**Hi-media**- Yeast extract, tryptone, agar powder

**Qualigens**- Hydrochloric acid.
2.2. Methods

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2.2.6.1. Isolation of RNA

2.2.6.2. RT-PCR
2.2.1. *Plasmodium falciparum* culture

2.2.1.1. Culture media

RBCs infected with *P. falciparum* (strains 3D7 or NF54) were maintained in complete RPMI medium. RPMI-1640 (Rosewell Park Memorial Institute) was originally developed for the *in vitro* cultivation of leucocytes. It is the most widely used medium for cultivation of *P. falciparum*. To make 1L of RPMI medium, 16.4g of RPMI-1640 (Hepes modified), 10g glucose and 2g sodium bicarbonate were dissolved in 1L of water. For complete RPMI-1640 medium, human serum (10%v/v) or Albumax II (0.5%w/v) was added. The medium was sterilized by passing through 0.22μm Millipore filter.

2.2.1.2. Processing of red blood cells

For RBC processing, 10ml of human blood was drawn and put in a sterile 50ml tube containing 2.5ml anticoagulant (ACD; For 100 ml, 0.0375M citric acid, 0.075M tri-sodium citrate and 0.15M dextrose). The cells were pelleted at 2,000rpm for 10min at 4ºC. The supernatant was discarded and the pellet was washed twice with incomplete RPMI (medium without human serum or Albumax II). Equal volume of complete RPMI medium was added to the pellet to generate a RBC stock of 50% hematocrit. The RBC stock was stored at 4ºC and used for around 20 days.

2.2.1.3. Initiation and maintenance of *P. falciparum* cultures

Frozen stock of *P. falciparum* culture was prepared by the Stockholm Sorbitol method (Schichtherle *et al.*, 2000). The freezing media consisted of 28% glycerol, 3% sorbitol and 0.65% NaCl. To make 250ml of freezing media, 180ml of 4.2% sorbitol in 0.9% NaCl was mixed with 70ml of glycerol and filter sterilized. The culture at 2 to 3% parasitemia, predominantly at ring stage, was transferred to a sterile falcon tube and pellet by centrifugation. To the 0.2ml pellet, 0.3ml of serum of complimentary blood group was added. The freezing media was then added drop by drop, while shaking gently. The culture was
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transferred to a sterile cryovial and stored in liquid nitrogen until use. To thaw the frozen culture, the vial was taken out from liquid nitrogen and thawed at 37°C for 1-2min. The culture was transferred into a falcon tube and volume was measured. To it 0.1X volume of 12%NaCl was added dropwise, while shaking the tube gently. The tube was left for 5min at RT. This was followed by addition of 10X volume of 1.6%NaCl slowly, dropwise. The tube was centrifuged at 20°C for 5min at 1500rpm. The supernatant was removed and 10X volume of complete media was added dropwise. The tube was again centrifuged at 1500 rpm for 5min at 20°C. The supernatant was removed and RBCs (at 5% hematocrit) and complete medium were added to the pellet and the culture was maintained in a 25cm² culture flask (Schichtherle et al., 2000).

For maintenance of parasite culture, parasitized RBCs were pelleted at 1000rpm for 5min at 4°C. The supernatant was discarded and a thin smear was made from the pellet and stained with Giemsa stain (Schichtherle et al., 2000). Percent parasitemia was determined by counting infected and non-infected cells under the microscope. The RBC stock (50% hematocrit) was diluted to 5% hematocrit in complete medium and added to an appropriate volume of culture such that the parasitemia was reduced to 1%. The culture was maintained in 60mm dishes as well as 25cm² and 75cm² culture flasks. The ideal gas phase required for growing the erythrocytic stages of malarial parasite comprises 2-5% CO₂ and 5-8% O₂ with a balance of N₂. The candle jar method of Jensen and Trager (Jensen and Trager, 1977) is the simplest way to achieve this gas phase. The cultures were placed in a desiccator and a lighted white candle was placed inside with the desiccator stop-cock open. As soon as the candle flame extinguished, the stop-cock was closed and the desiccator was placed in an incubator maintained at 37°C. The medium was changed every 24 hours and the cells were sub-cultured every fifth or sixth day.
2.2.1.4. Synchronization of *P. falciparum* cultures

Synchronization of cultures was carried out at high parasitemia when the parasites were predominantly in the ring stage. *In vitro* continuous culture of *P. falciparum* was synchronized from mixed stages by washing infected erythrocytes with 5% aqueous sorbitol (Lambros and Vanderberg, 1979). Normally erythrocytes are impermeable to sorbitol but the developing parasite affects a change in erythrocyte permeability to this sugar so that washing the erythrocytes in an aqueous sorbitol solution destroys the mature stages of the parasite. For synchronization, the parasite culture was pelleted. Three volumes of 5% D-sorbitol was added to the pellet. This was followed by incubation at 37°C for 10 min with intermittent shaking. The cells were pelleted down and the pellet was washed twice with incomplete medium. Erythrocytes (at 5% hematocrit) and complete medium were added to the pellet and the culture was maintained in a 25cm² culture flask. After sorbitol treatment, the cultures containing ring stage parasites were allowed to develop synchronously.

2.2.2. Amplification of IR-A and IR-B sector segments of the 35kb apicoplast genome

2.2.2.1. Isolation of total genomic DNA from *P. falciparum* culture

Total DNA was isolated from *P. falciparum* according to the method used by Qari et al. (1998). Briefly, *P. falciparum* cultures were lysed with 0.05% saponin and washed extensively with PBS. The DNA was released by adding 450μl of lysis buffer [50mM Tris-HCl (pH 8), 5mM EDTA, 100mM NaCl and 1% SDS]. 200μg of proteinase K was added and mixed by swirling. The reaction mixture was incubated at 42°C for 45 min. RNaseA (2μg) was added to the reaction, mixed and incubated at 37°C for 15 min to degrade RNA. The reaction mix was extracted with phenol-chloroform. To the aqueous phase, 0.04M NaCl and twice the volume of ethanol was added for precipitation. The DNA pellet was washed with 70% ethanol, dried and suspended in TE.
2.2.2.2. **PCR-amplification of segments of the apicoplast genome**

The 35kb apicoplast genome is divided into two sectors: the sector carrying IR-A inverted repeat region and sector carrying IR-B inverted repeat region. PCR-amplification of DNA fragments covering the IR-A (15.42kb) and IR-B (14kb) sectors was carried out by designing primer pairs to amplify the fragments in range of about 400bp to about 2.5kb (Fig. 2.1). IR-A and IR-B region was sectored into fragments. Primers covering the IR-A and IR-B fragments were designed using DNAstar software. These primers carried enzyme tags for cloning into appropriate vectors. Table I shows the sequences of primers used for amplification of plDNA fragments. Total *P. falciparum* genomic DNA isolated from infected blood cultures was used as template for PCR-amplification of apicoplast DNA. Table II shows the enzyme tags and PCR conditions for IR-A fragments and Table III shows the enzyme tags and PCR conditions for IR-B fragments.
Fig. 2.1. Position of fragments covering the IR-A and IR-B sectors of *P. falciparum* plDNA. The IR-A sector includes the inverted repeat region. Nucleotides of the IR-B sector are numbered after the end of the IR-B half of the inverted repeat (shown in dotted line).
Table I. Primers used for amplification of plDNA fragments.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Primer sequence (restriction enzyme tags are underlined)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A650</td>
<td>U: 5'-CCGGAATTCCATGAGTCGACGGGCGGTGTG-3' &lt;br/&gt; D: 5'-TGCTCTAGAAAGAATATCAAGCCGGAGGC-3'</td>
</tr>
<tr>
<td>A1820</td>
<td>U: 5'-CCGGAATTCCATGAGTCGACGGGCGGTGTG-3' &lt;br/&gt; D: 5'-TGCTCTAGAAAGAATATCAAGCCGGAGGC-3'</td>
</tr>
<tr>
<td>A1988</td>
<td>U: 5'-CCGGAATTCCATGAGTCGACGGGCGGTGTG-3' &lt;br/&gt; D: 5'-TGCTCTAGAAAGAATATCAAGCCGGAGGC-3'</td>
</tr>
<tr>
<td>A1288</td>
<td>U: 5'-CCGGAATTCCATGAGTCGACGGGCGGTGTG-3' &lt;br/&gt; D: 5'-TGCTCTAGAAAGAATATCAAGCCGGAGGC-3'</td>
</tr>
<tr>
<td>A1838</td>
<td>U: 5'-CCGGAATTCCATGAGTCGACGGGCGGTGTG-3' &lt;br/&gt; D: 5'-TGCTCTAGAAAGAATATCAAGCCGGAGGC-3'</td>
</tr>
<tr>
<td>A1967</td>
<td>U: 5'-CCGGAATTCCATGAGTCGACGGGCGGTGTG-3' &lt;br/&gt; D: 5'-TGCTCTAGAAAGAATATCAAGCCGGAGGC-3'</td>
</tr>
<tr>
<td>A1919</td>
<td>U: 5'-CCGGAATTCCATGAGTCGACGGGCGGTGTG-3' &lt;br/&gt; D: 5'-TGCTCTAGAAAGAATATCAAGCCGGAGGC-3'</td>
</tr>
<tr>
<td>B483</td>
<td>U: 5'-CCGGAATTCCATGAGTCGACGGGCGGTGTG-3' &lt;br/&gt; D: 5'-TGCTCTAGAAAGAATATCAAGCCGGAGGC-3'</td>
</tr>
<tr>
<td>B1624</td>
<td>U: 5'-CCGGAATTCCATGAGTCGACGGGCGGTGTG-3' &lt;br/&gt; D: 5'-TGCTCTAGAAAGAATATCAAGCCGGAGGC-3'</td>
</tr>
<tr>
<td>B2435</td>
<td>U: 5'-CCGGAATTCCATGAGTCGACGGGCGGTGTG-3' &lt;br/&gt; D: 5'-TGCTCTAGAAAGAATATCAAGCCGGAGGC-3'</td>
</tr>
<tr>
<td>B1308</td>
<td>U: 5'-CCGGAATTCCATGAGTCGACGGGCGGTGTG-3' &lt;br/&gt; D: 5'-TGCTCTAGAAAGAATATCAAGCCGGAGGC-3'</td>
</tr>
<tr>
<td>B1426</td>
<td>U: 5'-CCGGAATTCCATGAGTCGACGGGCGGTGTG-3' &lt;br/&gt; D: 5'-TGCTCTAGAAAGAATATCAAGCCGGAGGC-3'</td>
</tr>
<tr>
<td>B1880</td>
<td>U: 5'-CCGGAATTCCATGAGTCGACGGGCGGTGTG-3' &lt;br/&gt; D: 5'-TGCTCTAGAAAGAATATCAAGCCGGAGGC-3'</td>
</tr>
<tr>
<td>B1639</td>
<td>U: 5'-CCGGAATTCCATGAGTCGACGGGCGGTGTG-3' &lt;br/&gt; D: 5'-TGCTCTAGAAAGAATATCAAGCCGGAGGC-3'</td>
</tr>
</tbody>
</table>
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Table II. Enzyme tags & PCR Conditions for IR-A.

<table>
<thead>
<tr>
<th>Fragment size (bp)</th>
<th>Enzyme</th>
<th>Enzyme tags</th>
<th>Cycle conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRA 650</td>
<td>Promega Taq</td>
<td>EcoRI-Xba</td>
<td>Denaturation: 94°C, 1 min 50°C, 1 min - 35 cycles</td>
</tr>
<tr>
<td>IRA 1820</td>
<td>Promega Taq</td>
<td>EcoRI-Xba</td>
<td>Denaturation: 94°C, 1 min 50°C, 1 min - 6 cycles 55°C, 1 min - 29 cycles</td>
</tr>
<tr>
<td>IRA 1988</td>
<td>Promega Taq</td>
<td>EcoRI-Xba</td>
<td>Denaturation: 94°C, 1 min 44°C, 1 min - 6 cycles 50°C, 1 min - 29 cycles</td>
</tr>
<tr>
<td>IRA 1838</td>
<td>Promega Taq/ Platinum Taq</td>
<td>EcoRI-BamHI</td>
<td>Denaturation: 94°C, 1 min 40°C, 1 min - 6 cycles 50°C, 1 min - 29 cycles</td>
</tr>
<tr>
<td>IRA 1919</td>
<td>Platinum Taq</td>
<td>EcoRI-Xba</td>
<td>Denaturation: 94°C, 1 min 40°C, 1 min - 6 cycles 50°C, 1 min - 29 cycles</td>
</tr>
<tr>
<td>IRA 1967</td>
<td>Platinum Taq</td>
<td>EcoRI-Xba</td>
<td>Denaturation: 94°C, 1 min 40°C, 1 min - 6 cycles 50°C, 1 min - 29 cycles</td>
</tr>
<tr>
<td>IRA 1288</td>
<td>Promega Taq</td>
<td>BamHI</td>
<td>Denaturation: 94°C, 1 min 40°C, 1 min - 6 cycles 47°C, 1 min - 29 cycles</td>
</tr>
</tbody>
</table>

PCR Cycle Conditions standardized for ThermostarIII thermal cycler.
Table III. Enzyme tags & PCR Conditions for IR-B.

Pre-denaturation 94°C, 2 min  
Post-extension 72°C, 4 min  
Reaction volume 50 µl

PCR Cycle Conditions standardized for ThermostarIII thermal cycler.

<table>
<thead>
<tr>
<th>Fragment size (bp)</th>
<th>Enzyme</th>
<th>Enzyme tags</th>
<th>Cycle conditions</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRB 483</td>
<td>Promega Taq</td>
<td>XbaI - HindIII</td>
<td></td>
<td>94°C, 1 min</td>
<td>40°C, 1 min - 6 cycles 49°C, 1 min - 29 cycles</td>
<td>72°C, 1 min 30 sec</td>
</tr>
<tr>
<td>IRB 1308</td>
<td>Promega Taq</td>
<td>EcoRI-XbaI</td>
<td></td>
<td>94°C, 1 min</td>
<td>42°C, 1 min - 6 cycles 53°C, 1 min - 29 cycles</td>
<td>72°C, 2 min 30 sec</td>
</tr>
<tr>
<td>IRB 1426</td>
<td>Platinum Taq</td>
<td>EcoRI-BamHI</td>
<td></td>
<td>94°C, 1 min</td>
<td>40°C, 1 min - 6 cycles 50°C, 1 min - 29 cycles</td>
<td>72°C, 2 min 30 sec</td>
</tr>
<tr>
<td>IRB 1624</td>
<td>Platinum Taq</td>
<td>EcoRI-XbaI</td>
<td></td>
<td>94°C, 1 min</td>
<td>40°C, 1 min - 6 cycles 50°C, 1 min - 29 cycles</td>
<td>72°C, 2 min 30 sec</td>
</tr>
<tr>
<td>IRB 1639</td>
<td>Platinum Taq</td>
<td>EcoRI-BamHI</td>
<td></td>
<td>94°C, 1 min</td>
<td>40°C, 1 min - 6 cycles 50°C, 1 min - 29 cycles</td>
<td>72°C, 2 min 30 sec</td>
</tr>
<tr>
<td>IRB 1880</td>
<td>Platinum Taq</td>
<td>EcoRI-XbaI</td>
<td></td>
<td>94°C, 1 min</td>
<td>40°C, 1 min - 6 cycles 50°C, 1 min - 29 cycles</td>
<td>72°C, 2 min 30 sec</td>
</tr>
<tr>
<td>IRB 2435</td>
<td>Platinum Taq</td>
<td>EcoRI-XbaI</td>
<td></td>
<td>94°C, 1 min</td>
<td>40°C, 1 min - 6 cycles 50°C, 1 min - 29 cycles</td>
<td>72°C, 3 min</td>
</tr>
</tbody>
</table>
2.2.3. Cloning of the amplified fragments

2.2.3.1. Cloning strategy for fragments cloned in pUC18

PCR-amplified fragments A650, A1919, A1967, A1988, B1308, B1624, B1880 and B2435 were digested with restriction enzymes EcoRI and XbaI and cloned into pUC18 vector using standard procedures (Sambrook et al., 1989). Fig. 2.2(A) gives the cloning strategy for these fragments. Fragments B1426 and B1639 were digested with EcoRI and BamHI and cloned into the MCS of pUC18 as EcoRI-BamHI inserts [Fig. 2.2(B)]. The PCR-amplified fragment B483 was digested with XbaI and HindIII and cloned as an XbaI-HindIII insert into the MCS of pUC18 [Fig. 2.2(C)]. Fragment A1288 was cloned into the BamHI site of pUC18 [Fig. 2.2(D)].

2.2.3.2. Cloning strategy for fragments cloned in the pGEM-T easy vector

PCR-amplified fragments A1820 and A1838 were cloned into the pGEM-T easy vector (Promega) according to the manufacturer's instructions [Fig. 2.2(E)].

The sequence of all inserts was confirmed by restriction digestion (see results) and DNA sequencing (carried out at the National Institute of Immunology, New Delhi).

2.2.4. Apicoplast DNA purification and identification of replication origins by 5’ end-labeling

2.2.4.1. Purification of *P. falciparum* apicoplast DNA

plDNA-enriched DNA was purified according to the method of Yap et al. (1997) using the Qiagen plasmid mini preparation kit (Qiagen, USA). Infected RBCs from *P. falciparum* cultures were lysed with 0.05% saponin and washed extensively with PBS. The purified parasites were suspended in buffer P1, lysed with buffer P2 and neutralised with buffer P3.

Fig. 2.2(B). Cloning strategy for construction of pAB1426 and pAB1639.
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**Fig. 2.2(C).** Cloning strategy for construction of pAB483.

**Fig. 2.2(D).** Cloning strategy for construction of pAA1288.
Taq DNA Polymerase produces PCR products containing 3' A overhangs

pGEM-T easy vector carrying T overhangs

Fig. 2.2(E). Cloning strategy for construction of pAA1820 and pAA1838.
After centrifugation, proteinase K was added to the supernatant at a final concentration of 0.75 mg/ml and incubated for 2h at 37°C. The sample was passed through a Qiagen tip-20 column previously equilibrated with QBT buffer. The column was washed with QC buffer and the extra-chromosomal DNA was eluted with buffer QF preheated to 65°C. The DNA was precipitated with isopropanol, washed with 70% ethanol, dried and suspended in TE [10mM Tris (pH 8), 1mM EDTA].

2.2.4.2. 5' end-labeling of nascent strands and enrichment of replication intermediates

Labeling of nascent DNA strands followed by enrichment of replication intermediates was carried out as described by Lu, Kunnimalaiyan and Nielsen (Lu et al., 1996) and Little and Schildkraut (1994). Apicoplast DNA was dephosphorylated using calf intestinal alkaline phosphatase (CIAP). Proteinase K (50μg/ml) was added to the sample and incubated at 37°C for 1h. The sample was subjected to phenol-chloroform extraction and ethanol precipitation. This was followed by labelling with [γ-32P]-ATP (100μCi) in the presence of 10U of polynucleotide kinase at 37°C for 1h (Fig. 2.3). The end-labeled DNA was digested with suitable restriction enzymes. The labeled and digested DNA was loaded onto a benzoylated naphthoylated DEAE-cellulose (BND-cellulose) column that had been pre-equilibrated with 0.3M NET8 [0.3M NaCl, 10mM Tris-HCl (pH 8), 1mM EDTA]. The column was washed three-times with 0.3M NET8 followed by three washes with 0.8M NET8 [0.8M NaCl, 10mM Tris-HCl (pH 8), 1mM EDTA]. This fraction contains linear double-stranded DNA and is termed the ‘salt wash’. DNA enriched in replication intermediates was then eluted by washing the column three times with 1M NET8/1.8% (w/v) caffeine [1M NaCl, 10mM Tris-HCl (pH 8), 1mM EDTA, 1.8% (w/v) caffeine].
Fig. 2.3. Schematic representation of the method of 5' end-labeling of nascent DNA. (A) Apicoplast DNA purified at the late trophozoite/early schizont stages may follow the bi-directional or D-loop mechanism of replication. (B) De-phosphorylation of the 5' ends of purified apicoplast DNA by calf intestinal alkaline phosphatase (CIAP). (C) T4-polynucleotidyl kinase in presence of γ-32P ATP end labels the nascent DNA. (D) The end-labeled nascent DNA is digested with restriction enzymes. Dotted lines symbolize the sites for restriction enzymes. Only fragments originating from a replication origin carry the 5' end labels. (E) The 5' end-labeled nascent DNA is purified by BND-cellulose chromatography, a method for the enrichment of replication intermediates. (F) The nascent DNA eluted in the caffeine fraction is used as probe in Southern hybridizations thus enabling identification of DNA replication initiation sites.
The 'salt' and 'caffeine' washes were precipitated with ethanol, washed with 70% ethanol, dried and suspended in TE. DNA from the 'salt wash' and 'caffeine wash' was analysed for radioactivity (Cerenkov counting). The latter was denatured and used as probe in Southern hybridization.

2.2.4.3. Southern hybridization of restriction-digested plasmid constructs carrying plDNA inserts
Plasmid constructs carrying the 35kb plDNA sequences were digested with restriction enzymes to release their respective inserts. The digested products were electrophoresed on a 0.8% or 1.4% agarose gel in 1XTAE buffer (diluted from 50X TAE; For 1L, 242g Tris-HCl, 57.1ml glacial acetic acid and 100ml of 0.5M EDTA). The gel was then depurinated in 0.25N HCl for 15 min, denatured in 1.5M NaCl and 0.5N NaOH for 15 min followed by neutralisation in 1M ammonium acetate. The DNA was transferred onto nylon membrane (Hybond N+) in 1M ammonium acetate for 16h. The membrane was baked at 80°C for 2h. The 5' end-labeled nascent DNA ('caffeine wash') was used as probe for hybridisation (Sambrook et al., 1989). The nylon membrane was washed and exposed to X-ray film for autoradiography. Densitometric analysis of the autoradiogram was carried out using Imagemaster 1D Elite software (Amersham Biosciences).

2.2.5. Measurement of in vivo ori activity by competitive PCR
2.2.5.1. Purification of nascent DNA for competitive PCR
Total *P. falciparum* DNA was isolated from cultures from ten T75 flasks at ~10% parasitemia containing parasites at the late trophozoite-early schizont stages. Total parasite DNA was isolated by phenol/chloroform extraction (Qari et al., 1998). Isolation of nascent DNA was carried out by sucrose gradient fractionation followed by further size selection of the fractionated DNA by agarose gel electrophoresis (Kumar et al., 1996; Tao et al., 1997; Habib and Hasnain, 2000). Total parasite DNA was denatured by a 10min
incubation in boiling water and size separated on 16ml of 5 to 30% continuous neutral sucrose gradient (~150μg of DNA per gradient) for 18 to 20h at 26,000 rpm in a Beckman SW28 rotor at 15°C. Sucrose gradients were prepared in 10mM Tris-HCl (pH 8), 1 mM EDTA, and 0.3M NaCl. The bottom of the tube was punctured and 500μl fractions were collected from each tube. Fractions containing 0.3kb to 1.5kb segments of nascent DNA were pooled and dialyzed against Tris-EDTA (0.5M Tris (pH8), 0.01M EDTA) for at least 8h. DNA was precipitated with sodium acetate and ethanol, rinsed with 70% ethanol, dried, and suspended in TE. Further size selection was performed by fractionating the nascent DNA on a 1% preparative agarose gel and eluting 0.3 to 1.5kb segments of DNA from the gel. After purification, the concentration of this DNA was determined and the preparation was used as template in competitive PCRs.

2.2.5.2. PCR-amplification and competitor construction
Primers used for competitor construction and competitive PCR analysis of the 325bp and 251bp ori regions (regions I and II, respectively) as well as primers used to amplify the 332bp and 317bp control non-orI regions (regions III and IV, respectively) are shown in Table IV. Competitor construction for each of these regions was carried out as described by Diviacco et al. (1992) and Habib and Hasnain (2000). Four specific oligonucleotides (two external primers, P1 and P2, and two internal primers P3 and P4) were synthesized for each region to be amplified [Fig. 2.4(A)]. The external primers were designed to amplify DNA regions in the range of 150 to 350bp. The sequence of the upper (P1) and lower (P2) external primers is identical to the genomic region to be amplified.
Table IV. Primer sequences and PCR product lengths of ori and control regions. (C) denotes the complementary strand sequence.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Primer coordinates (nt)</th>
<th>Sequence of the primer with tags in bold</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>P1: 1635-1655&lt;br&gt;P2: 1933-1959 (C)&lt;br&gt;P3: 1819-1838 (C)&lt;br&gt;P4: 1839-1860</td>
<td>P1: 5'-CTGAGCTAGGATCAAAGCTGC-3'&lt;br&gt;P2: 5'-CGAAATAGGTAACGCACTAAA&lt;br&gt;P3: 5'-ACCTGCAGGGATCGTGCACCG&lt;br&gt;AATGCTTTAACCACCTAAG-3'&lt;br&gt;P4: 5'-CTCGACGGGATCCCTGCAGGTC&lt;br&gt;TGTTAACGAAATACACATAG-3'</td>
<td>Genomic, 325&lt;br&gt;Genomic, 345</td>
</tr>
<tr>
<td>II</td>
<td>P1: 3974-3994&lt;br&gt;P2: 4202-4224 (C)&lt;br&gt;P3: 4080-4099 (C)&lt;br&gt;P4: 4100-4120</td>
<td>P1: 5'-GATTGACCTCGCCGATGTC-3'&lt;br&gt;P2: 5'-GCTTCATAGGGTCTGTTCTG&lt;br&gt;CC-3'&lt;br&gt;P3: 5'-ACGTCCAGGGATCGTGCACCG&lt;br&gt;ACAAGGAATTTGCTACC-3'&lt;br&gt;P4: 5'-CTCGACGGGATCCCTGCAGG&lt;br&gt;TGTTAACGAAATACACATAG-3'</td>
<td>Genomic, 251&lt;br&gt;Genomic, 271</td>
</tr>
<tr>
<td>III</td>
<td>P1: 14382-14411&lt;br&gt;P2: 14686-14713 (C)</td>
<td>P1: 5'-TTATAAAAAATGTTATCATGTA&lt;br&gt;TAAAGA-3'&lt;br&gt;P2: 5'-ATATCTATACCATCCATCAAC&lt;br&gt;CAGG-3'</td>
<td>Genomic, 332</td>
</tr>
<tr>
<td>IV</td>
<td>P1: 10213-10236&lt;br&gt;P2: 10506-10527 (C)</td>
<td>P1: 5'-CAGATTTGAACTGATACACAT&lt;br&gt;GG-3'&lt;br&gt;P2: 5'-CATGTCTAAAGGATCCGAC&lt;br&gt;C-3'</td>
<td>Genomic, 317</td>
</tr>
</tbody>
</table>


Fig. 2.4(A). Schematic representation of the competitive PCR method.

Fig. 2.4(B). Competitive PCR analysis. A fixed amount of nascent DNA template with 10-fold serial dilutions of competitor for each region is taken in the presence of P1 and P2 primers. Both the competitor and the template compete for the same primer set and thus amplify at the same rate. The ratio between the final amplification products of the two species is evaluated at each time point and is a precise reflection of the ratio between the initial amounts of the two templates. This ratio is used to evaluate the amount of unknown nascent DNA template.
The upper (P4) and lower (P3) internal primers have 3' ends identical to contiguous sequences on the upper and lower genomic strands, respectively, and their 5' ends carry a 20 nt tag. The 20 nt tags of the P4 and P3 primers are complementary to each other and unrelated to the target sequence to be amplified. Competitor DNA segments carrying the corresponding genomic sequence with the addition of 20 extra bp in the middle were constructed for each primer set in a three-step process (Habib and Hasnain, 2000).

Firstly, P1-P3 and P2-P4 amplification products were run on a 8% polyacrylamide gel. The two bands were touched with the needle and soaked in a tube containing 30μl of MQ water. 5μl of this was included in a standard PCR amplification mixture containing the two external primers P1 and P2. In order to allow the formation of the hetero-duplex product annealed at the complementary sequence with the 5' protruding ends, the reaction was denatured at 94°C for 1 min and then the temperature was slowly lowered to 50°C in a period of 10 min (Habib and Hasnain, 2000). After a further incubation for 2 min at 50°C, the reaction was incubated at 72°C for 5 min and was amplified by using the PCR conditions: 30 cycles, 94°C for 30 sec, 47°C for 1 min, 72°C for 1 min. One or more subsequent re-amplification steps of the full-length competitor were needed to enrich for the competitor product and allow its quantification by radioactive labeling. All amplification reactions were carried out in an advanced version of thermostarIII thermal cycler (Upadhyay, 1999).

2.2.5.3. Quantitation of competitors
Competitor template for each DNA region was quantified by measuring the amount of incorporated [α-32P]-dATP in a competitor re-amplification PCR cycle. The competitor PCR amplification mixture (50μl) contained the standard amount of cold dATP (10nmol) and 0.2 μl (0.57pmol) of [α-32P]-dATP (Jonaki, India) (3,500Ci mmol⁻¹ and 10mCi ml⁻¹), corresponding to 1.34 x 10⁷cpm as measured by
Cerenkov counting in a β-counter. The amplification products were resolved on an 8% polyacrylamide gel, and the labeled competitor band was eluted in 100μl of water. Five microlitre of the eluted DNA was counted and the concentration of competitor (number of molecules per microlitre) was determined from the final specific activity of [α-32P]dATP and the number of nucleotides incorporated. Dilutions of this quantified competitor preparation were used as template in competitive PCRs.

2.2.5.4. Competitive PCR experiments

Competitive PCR experiments were carried out for each region by first using a fixed amount of nascent DNA template with 10-fold serial dilutions of the corresponding competitor in the presence of primers P1 and P2. The range within which the point of equivalence between competitor and template lay was thus determined [Fig. 2.4(B)]. Further dilutions of competitor within this range were then used in similar PCR reactions. Competitive PCR for the 325bp (region I) [Fig. 3.20 in results] and 251bp (region II) [Fig. 3.20 in results] segments was carried out in 30 cycles with the following conditions: denaturation, 94°C, 30 sec; annealing, 47°C, 1 min; extension, 72°C, 1min. PCR conditions for amplification of the control non-ori region IV (317bp) was same as above two but for region III (332bp) the conditions were: denaturation at 94°C for 30 sec, annealing at 41°C for 1 min and extension at 68°C for 1min (Platinum pfX polymerase from invitrogen was used for this reaction).

2.2.6. RNA isolation and RT-PCR

2.2.6.1. Isolation of RNA

The infected RBCs from P. falciparum cultures were lysed with 0.05% saponin and washed extensively with PBS. To the pellet 500μl of solution D (4M GITC, 25mM sodium acetate, 0.5% sarcosyl, 0.1Mβ-M.E., DEPC water) was added. After homozenisation with a 22 gauge
needle, 50μl of sodium acetate, 500μl of water saturated phenol and 100μl chloroform-isoamylalcohol in the ratio 49:1 was added and mixed well. This was followed by incubation on ice for 15 min. After centrifugation at 12000rpm for 20 min at 4°C, the aqueous phase was collected and 500μl of isopropanol was added for precipitation. The sample was incubated at ~20°C for 3h followed by centrifugation at 12000rpm. The pellet was suspended in 300μl solution D, 300μl of isopropanol was added to it and left overnight at ~20°C. The pellet was washed with 70% ethanol, dried and suspended in DEPC-treated water.

2.2.6.2. RT-PCR

The RNA sample was treated with 2μl DNase (GIBCO-BRL (Invitrogen); concentration 1U/μl) to remove any DNA contamination. After incubation at RT for 15 min, DNase was inactivated by adding 25mM EDTA and incubation at 65°C for 10min. RNA was precipitated with sodium acetate and ethanol, washed with 70% ethanol, dried and suspended in DEPC-treated water. This RNA sample was used for RT-PCR reaction. For the RT-PCR reaction, superscript™ one step RT-PCR kit (Invitrogen) was used. The reaction consisted of the reaction mix, template RNA and GINT(U) [primer sequence: 5'-CGC GGA TCC ATG GCT AAT ATT AAT TCA ATC ATC TTT AGT G-3'] and GINT(D) [primer sequence: 5'-CGC GGA TCC TTA AGG AAT AGT TAC TGT TAA AGA ATT-3'] primers. The first step was denaturation at 94°C for 2 min. The reaction was allowed to cool down to 40°C. 1μl reverse transcriptase/Taq polymerase mix was added to the reaction. The reaction was incubated at 40°C for 30 min for reverse transcription to take place. After the cDNA synthesis, DNA was amplified using following PCR conditions: 3 cycles at 94°C for 30 sec, 40°C for 1 min, 72°C for 90 sec and 42 cycles at 94°C for 30 sec, 50°C for 1 min, 72°C for 90 sec.
For the determination of the mono-/polycistronic nature of ORF470, GINT(U), GINT(D) and \textit{rpoB}(D) primers were used. \textit{rpoB}(D) primer sequence: \texttt{5'-CTG AAT AGT ATC AAT AGA ATT TAT ATT TAT ATT AG-3'}. 