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The gene encoding topoisomerase II from *Plasmodium falciparum*

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ABSTRACT

The gene for topoisomerase II has been isolated from genomic libraries of strain K1 of the human malarial parasite, *Plasmodium falciparum*. The sequence reveals an open reading frame of 4194 nucleotides which predicts a polypeptide of 1398 amino acids. There are apparently no introns. The sequence is present as a single copy which has an identity of 47.4% and a similarity of 65.4% with its human homologue. Sequences conserved in topoisomerase II from other species are present in Pftopoisomerase II but in addition it has two adjacent asparagine-rich insertions which are unique to it. We have also detected asparagine-rich regions in the gene for PfDNA polymerase α. The gene for Pftopoisomerase II has been localised to chromosome 14 and northern analysis reveals a transcript of 5.8kb. Two independent antisera raised in mice against glutathione-S-transferase fusion proteins containing the amino terminal portion of the malarial protein detect a weak band on western blots at about 160kDa, the expected size of the protein. Use of the same antisera for immunofluorescence analysis suggests that the protein is present at all stages of intraerythrocytic growth of the parasite.

INTRODUCTION

Class II topoisomerases act by cleaving both strands of a DNA double helix, passing an unbroken DNA duplex through the gap produced before resealing the gap (Reviews see 1,2). As a result they are able to relieve torsional stresses and resolve topological constraints resulting from DNA replication. Topoisomerase II is known to be essential for the completion of chromosomal segregation at mitosis in yeast (3,4). It also appears to have important structural functions in the cell and is present throughout the cores of metaphase chromosomes of HeLa cells (5). It is also present in meiotic chromosomes of chicken spermatocytes (6) and yeast (7). The evidence suggests that it facilitates the condensation of yeast chromosomes *in vivo* (8) and chicken erythrocyte chromosomes *in vitro* (9). Topoisomerase II associates with A/T-rich regions of the DNA and is found in preparations of the nuclear matrix and scaffold from chicken interphase nuclei (10) and in *Drosophila* (11).

Topoisomerase II has been reported to be present at high levels in rapidly dividing cells (12,13) and a number of anti-cancer drugs are known which act as topoisomerase II poisons (2). These either intercalate into the DNA (e.g. m-AMSA and ellipticine) or they act by trapping the cleavable complex formed when each 5′ terminus of the cleaved duplex becomes covalently linked to a molecule of the enzyme through a specific tyrosyl residue. This can be demonstrated by denaturing the complex with alkali or SDS whereupon protein-associated strand breaks appear. The trapped cleavable complexes are important contributors to cell death although precisely how they do this is not yet known and other cellular factors appear to be important (summarised in 1). The malarial parasite also divides rapidly after it enters the liver of the human host and after invasion of the erthyrocyte and attempts have been made to evaluate topoisomerase II poisons as potential antimalarial agents. Recently, interest has been focused on the 9-anilinoacridines which inhibit parasite metabolism (assayed by the incorporation of ^{3}[H] hypoxanthine) and inhibit the decatenation of kinetoplast DNA by parasite extracts (14). They are related structurally to the synthetic antimalarial, pyronaridine, which has already been studied extensively and has undergone clinical trials (15). From a study of a number of 9-anilinoacridine derivatives it was concluded that hydrophilicity and a high ionisation constant are the important factors in determining activity. One of the active derivatives, 3,6 diamino-1′-amino-9-anilinoacridine, was highly selective in its action inhibiting malarial topoisomerase II activity at only 1/600th the level needed to inhibit mammalian topoisomerase II to the same extent.

As part of a wider program to evaluate the possibilities of using the parasite's DNA replication apparatus as a drug target we have set about isolating genes encoding its component polypeptides (16,17,18). In the present report we describe the cloning and

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the characterisation of the parasite gene for topoisomerase II. Work is currently underway to express the replication proteins heterologously to facilitate a detailed biochemical study of their properties.

MATERIALS AND METHODS

Parasite culture

The K1 isolate of *P.falciparum* was used throughout (19). It was cultured using the method of Trager and Jensen (20) as modified by Zolg *et al.* (21). Cultures were synchronised as required using the method of Lambros and Vandenberg (22).

DNA and RNA, isolation and blotting

The methods used for nucleic acid isolation (23,24,25), Southern (26) and Northern blotting (24) have already been fully reported elsewhere.

Isolation of the gene for topoisomerase II

The sequence *MIMTDQD* is highly conserved in other eukaryotic topoisomerase II genes (27,28,29). This was used to design the oligonucleotide probe ATGAT(A/C/T)ATGAC(A/G/C/T)GA-(C/T)CA(G/A)GA. The oligonucleotide was end labelled and used to probe a library of *Hin*dIII digested genomic parasite DNA constructed in λNM1149. The first fragment to be isolated contained the 5′ end of the gene and several hundred bases of 5′ flanking DNA. Overlapping clones were obtained by screening genomic DNA libraries constructed in both λNM1149 and λgt11 with oligonucleotides, random labelled gene fragments or PCR products. The isolated fragments were subcloned into pUBS (30) or pUC19 (31) and sequenced on both strands by the Sanger and Coulson method (32) using Sequenase Kit version 2.0 (USB). The University of Wisconsin Genetics Computer Group (UWGCG) programs were used for sequence comparisons (33).

Chromosome blots

Established methods of pulsed field gel electrophoresis (16) were used to separate the chromosomes of *P.falciparum*. Chromosome blots were prepared using Genescreen Plus membranes following the manufacturers' instructions.

Fusion protein constructs and antibody production

The polymerase chain reaction was used to generate a product with a 5′ *Bam*HI recognition site. The product encoded the amino acids 6 to 144 inclusive of the Pftopoisomerase II predicted protein. This excludes two asparagine-rich inserts in the sequence (see Fig. 2 and Results). After cleavage with *Bam*HI, the product was cloned into *Bam*HI- and *Sma*I-digested pGEX2 in frame with the glutathione-S-transferase (GST) coding sequence (34). Cloning junctions were confirmed by sequencing and the construct was transformed into *E.coli* BL21 cells for expression of the fusion product. The production of the fusion protein was inferred from the appearance, after induction with 0.1mM IPTG, of a major product of the anticipated size (42kDa) and the simultaneous disappearance of the band for GST (27kDa) on Coomassie Blue-stained SDS polyacrylamide gels. Approximately 20μg of insoluble fusion protein were emulsified in a mixture of 0.15ml phosphate-buffered saline (PBS) and 0.15ml complete Freunds adjuvant and used to immunise mice. The mice were boosted at two and four weeks after the initial immunisation. Individual sera prepared from these mice were used for immunofluorescence analysis of fixed, synchronised parasites and for western blot studies.

Serum preparation and Western blots

Before use, the mouse anti-Pftopoisomerase II serum was preadsorbed to GST bound to glutathione-agarose beads (Sigma) in order to remove antibodies recognising GST epitopes. Removal of these antibodies was verified by western blot analysis using purified GST. Western blot analysis was performed as described earlier (16). Western blots of parasite extracts which had been reacted with mouse anti-Pftopoisomerase II serum were also analysed using the enhanced chemiluminescence system (ECL Amersham) incorporating anti-mouse IgG second antibody-linked horseradish peroxidase.

Immunofluorescence studies

Acetone-fixed, parasitised erythrocytes from synchronous and asynchronous cultures were incubated with anti-Pftopoisomerase II serum for one hour at high humidity. After washing to remove unadsorbed antibodies the slides were treated with a fluorescin isothiocyanate (FITC) conjugate of goat anti-mouse IgG (Sigma). The slides were also counterstained with the nuclear-specific stain DAPI. Detection of intact parasites in samples taken soon after the synchronising treatment is often made difficult by cellular debris. To overcome this, synchronised material prepared as described above was also reacted with a polyclonal rabbit anti-*P.falciparum* parasitophorus vacuole membrane antibody and a rhodamine (RITC) conjugate of anti-rabbit IgG.

RESULTS

The structure of the topoisomerase II gene from *P.falciparum*

The initial clone containing the 5′ coding and flanking sequences of topoisomerase II was obtained as a 2.6kb fragment from a genomic *Hin*dIII DNA library in λNM1149. Overlapping clones were isolated from a series of genomic DNA libraries. Their relationship to each other is given in the genomic map of the region (Fig. 1). Both strands of the open reading frame were completely sequenced. Pftopoisomerase II is encoded by an open reading frame of 4194bp which predicts a protein of 1398 amino acids with an anticipated molecular weight of 160.97 kDa. Application of the criteria of Li *et al.* (35) suggests there are no introns.

Figure 2 shows regions of an alignment of the predicted amino acid sequence from Pftopoisomerase II with homologous sequences from a number of other species. Overall the parasite and human topoisomerase II reveal an identity of 47.4% and a

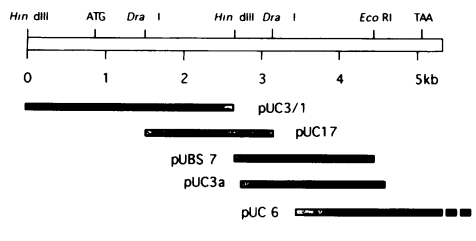

Figure 1. Diagrammatic representation of the *P.falciparum* topoisomerase II gene with selected restriction enzyme sites. The clones used for sequencing are given. pUC3A was obtained from a library of sheared genomic fragments.

similarity (identical plus conserved residues) of 65.4%. The figures for the parasite and *S.cerevisiae* are similar (44.4% and 62.2% respectively). The degree of conservation is generally greater in the amino terminal two-thirds of the coding sequences and falls off markedly towards the carboxy termini.

A number of features have been identified in the topoisomerases II of other organisms which are implicated in specific aspects of its function. These are conserved in the Pftopoisomerase II also. Substitution of glycine 144 in the yeast sequence by leucine results in loss of ATPase activity (36). The corresponding residue in Pftopoisomerase II is glycine 147. This region is conserved in all topoisomerase II molecules and is highly homologous with the ATPase domain of the gyrase B subunit of *Escherichia coli*. The tyrosine residue at position 830 in Fig. 2 is conserved in all species so far studied. It corresponds to the residue forming the transient covalent link with the 5′ ends of the severed DNA duplex (37) stabilising the 'gate' through which the unbroken duplex is passed. The predicted protein product of the Pftopoisomerase II gene differs from the sequences of all other species so far studied in its possession of two asparagine-rich insertions starting at position 267 (Fig. 2). The presence of blocks of inserted amino acids, often containing repetitive sequences, and the occurrence of asparagine-rich regions is well documented for other *Plasmodium* proteins (38) and, in particular, we previously identified asparagine-rich regions in the predicted parasite DNA Polymerase α (18). Here the two features coincide. The presence of nucleotides encoding this region in the mature message was demonstrated using cDNA-directed PCR with oligonucleotides flanking the insertions. Both cDNA and genomic DNA gave PCR products of identical size. As a control the same RNA sample was used to prepare cDNA subtending the intron of the PfDNA Polα gene (18). PCR products generated from this cDNA and from genomic DNA using the appropriate flanking oligonucleotide primers gave evidence of intron removal as expected (Fig. 3).

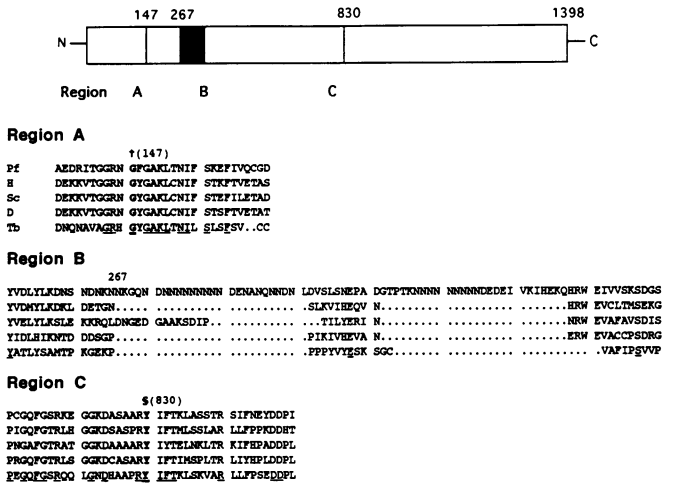

Figure 2. Plan of the predicted topoisomerase II from *P.falciparum* showing the position of the inserts and of the residues implicated in functional activity. The segments concerned are given in greater detail beneath in a UWGCG pileup analysis comparing topoisomerase II sequences from *P.falciparum* (Pf), Man (H), *Saccharomyces cerevisiae* (Sc), *Drosophila melanogaster* (D) and *Trypanosoma brucei* (Tb). Universally conserved residues are underlined. For further explanation of the residues at positions 147(†) and 830(§) see text.

Chromosomal location and transcript size

Pulsed field gel electrophoresis was used to separate the chromosomes of the parasite and blots were prepared and probed with a random-labelled 0.77kb *Bam*HI/*Hin*dIII fragment from the 3′ end of clone pUC3/1. The gene is located on chromosome 14 and this was confirmed by stripping the blot and reprobing with the chromosome 14 marker aldolase (39) (Fig. 4). Southern blot analyses suggest that the gene is present as a single copy (data not shown).

The same labelled fragment was used to probe a blot of poly(A)$^+$RNA prepared from unsynchronised cultured parasites. A band at ~5.8kb was recognised. This is approximately 2kb longer than the open reading frame. Similar size differences

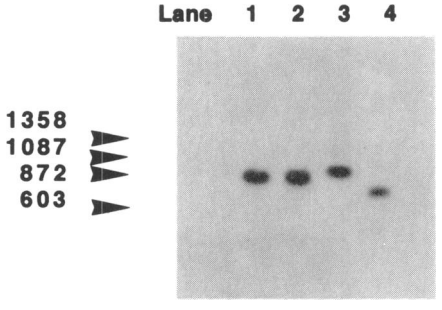

Figure 3. Results of cDNA- and genomic DNA-directed PCR experiment to confirm the presence in the transcript of sequences encoding the asparagine-rich insertions. PCR products were separated on a 1% agarose gel, southern blotted and probed with internal oligonucleotides flanking the regions of interest. Lane 1; gDNA-directed PCR product covering the asparagine-rich inserts. Lane 2; the same PCR product derived from cDNA. Lanes 3 and 4; Control reactions with gDNA (Lane 3) and cDNA derived from the same RNA preparation (Lane 4) covering a region containing the intron of the DNA Polα gene of *P.falciparum*. The difference in band size represents the result of splicing out the intron of 204 nucleotides.

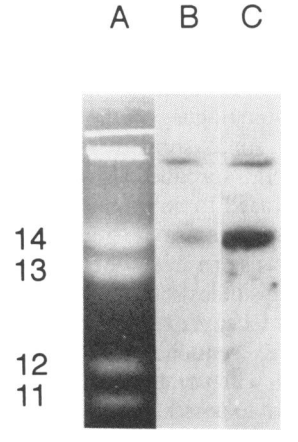

Figure 4. Chromosomal location of the coding sequence of Pftopoisomerase II. Panel **A**; Chromosomes of strain K1 separated and visualised with ethidium bromide before blotting Panel **B**; Chromosome blot probed with random-labelled *Bam*HI/*Hin*dIII fragment of clone pUC3/1. Panel **C**; The same blot stripped and reprobed with the chromosome 14 marker, aldolase. The relevant chromosomes are numbered.

between open reading frame and corresponding message have been noted in the case of PCNA (17) and DNA Polδ (16).

Use of antibodies in Western blots and immunofluorescence assays

Two separate antisera were raised. In both cases they recognised fusion protein in *E.coli* extracts strongly but showed a weak response towards GST. The latter was completely abolished by preadsorbing the antisera with immobilised GST. The reaction of the preadsorbed antisera with fusion protein was unchanged. We therefore believe the antisera are recognising topoisomerase II epitopes in the fusion protein. Both antisera revealed several weak bands in blots of size-fractionated parasite proteins. Although these did not always correspond in size, both antisera detected a weak band at about 160kDa, the anticipated size of Pftopoisomerase II (data not shown). The same antisera were used to probe fixed parasites in order to try and locate topoisomerase II within the cell and to determine when it appears during the intraerythrocytic development of the parasite.

Synchronised and unsynchronised material was used to do this. Synchronisation was achieved using two sequential sorbitol treatments separated by 16 hours. Immediately following the second sorbitol synchronisation most parasites appeared, as expected, as ring forms. Eight hours afterwards, trophozoites had increased to approximately 80% of the intraerythrocytic forms. In this synchronised sample the increase in schizonts was slightly delayed, taking place at between 20 and 24 hours after the second synchronisation (between 36 and 40hr post invasion). Both antisera stained the parasites at all stages in their development. The same result was obtained from non-synchronised material. No obvious difference existed in the intensity of the fluorescence at different stages of development. The observations suggest that the antigen distribution coincides with the bodies staining with DAPI (see Fig. 5) and we propose that Pftopoisomerase II is present in the nuclei of the parasite at all stages of its intraerythrocytic development.

DISCUSSION

We have described an open reading frame which encodes a polypeptide with all the features of a type II DNA topoisomerase. The sequence appears to be present as a single copy located on chromosome 14 of strain K1 and we believe it to be the gene for the malarial homologue of the enzyme. Immunological evidence provides some support for this conclusion. Two independent antisera, produced by mice inoculated with recombinant GST fused to an amino terminal fragment of the putative DNA topoisomerase II, recognised a protein of the expected size in western blots of whole parasite extracts. Immunofluorescence studies show that they also bind to a protein which appears to be located mainly in the parasite nucleus. Final evidence that this sequence is correctly identified as Pftopoisomerase II will come when the overexpressed gene product can be shown to possess topoisomerase II activity *in vitro*. These experiments are in progress.

The predicted amino acid sequence described shows high levels of homology with other known class II topoisomerases. The conserved motifs present in the topoisomerases II found in other species are all present in the *P.falciparum* sequence in the same spatial arrangement. The only distortion of precise colinearity is the result of two asparagine-rich insertions which are present only in the *Plasmodium* protein. Insertions, with or without repetitive regions, and asparagine-rich regions are not uncommon features of the proteins of the malarial parasite (38) and have been found in another enzyme associated with DNA replication, DNA Polymerase α (18). At present, their functional significance is unknown.

The expression of topoisomerase II has been the subject of several studies. Increased levels of topoisomerase II often correlate with rapid cell division. Rapidly dividing *Drosophila* K_c cells in culture (for example) had higher levels of topoisomerase II transcript than non-dividing cells (40) and Heck *et al.* (41) have reported differences in the levels of topoisomerase II protein in cultured chicken lymphoblastoid cells through the cell cycle. However marked changes in topoisomerase II levels are not always found. They do not vary greatly in budding yeast (42) although, in this case, the extent of casein kinase II-dependent phosphorylation of topoisomerase II does vary and increases markedly as the cells enter mitosis. Topoisomerase II is activated by phosphorylation (reviewed in (43)) and the importance of topoisomerase II activity at mitosis in yeast is emphasised by the observation that topoisomerase II temperature-sensitive mutants held at the restrictive temperature undergo a rapid loss of viability at this point in the cell cycle (4). From the present work, it appears that topoisomerase II is present at all stages of intraerythrocytic growth of the parasite as judged by the interactions with two different antisera raised against the fusion protein. In this respect Pftopoisomerase II appears to differ from the other replication proteins we have been able to study. Levels of PCNA, for example, increase rapidly just before schizogony from virtually undetectable levels in early trophozoites and late rings (17) and the same pattern appears to hold from preliminary experiments with DNA polymerase δ (McAleese, unpublished data). At this point we have still to assess the importance of phosphorylation in regulating the activity of the enzyme, but it should be noted that the Pftopoisomerase II sequence possesses a number of putative substrate sites for phosphorylation by a range of protein kinases.

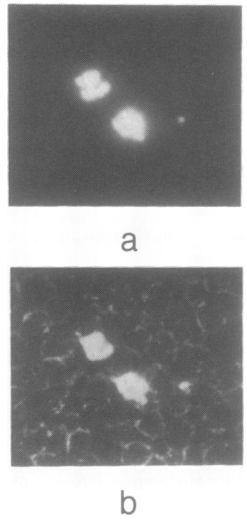

Figure 5. Schizont and trophozoite stages of parasites fixed and stained with DAPI (**a**) or antiserum raised against the fusion protein (**b**).

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