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Molecular Identification of a cDNA Encoding the Hypoxanthine-Guaninephosphoribosyltransferase (HGPRT) from Rodent Malaria Strain of *Plasmodium berghei*

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Abstract

The hypoxanthine-guaninephosphoribosyltransferase (HGPRT) enzyme of *Plasmodium berghei,* plays a key role in the salvage of preformed purine nucleotides from parasite-infected erythrocytes. Since *P. berghei* cannot synthesize purines *de novo*, development of inhibitors specific for the parasite HGPRT should be an effective method of chemotherapy. This gene has been annotated as encoding PRTase activity and proposed as essential for survival of the *P. berghei*. The aim of this work is to identify the HGPRT from *P. berghei* using bioinformatic tools in searching the FullMal database for EST sequences for a cDNA clone with full length *HGPRT*, design specific oligonucleotides primers and then isolate PbHGPRT from the cDNA clone. The PCR analysis produced an amplicon of 696 bp which is in agreement with the predicted size from the nucleotides sequence deduced from the HGPRT cDNA open reading frame. The significance of the above results is discussed in the light of existing literature.

Keywords

Plasmodium berghei, HGPRT, Phosphoribosyltransferase, Bioinformatics, EST, cDNA

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1. Introduction

Malaria remains one of the world's most devastating infectious diseases. Drug resistance to all classes of antimalarial agents has now been observed, highlighting the need for new agents that act against novel parasite targets. The complete sequencing of the *Plasmodium falciparum* genome has allowed the identification of new molecular targets within the parasite that may be amenable to chemotherapeutic intervention.

Malarial parasites are incapable of *de novo* purine biosynthesis during the intra-erythrocytic stages of their life cycle in the mammalian host. They therefore rely on the host to provide the necessary free pre-formed purines and have an efficient purine salvage machinery of their own (Büngener and Nielsen, 1968; Gutteridge and Trigg, 1970; Gutteridge and Coombs, 1977). Enzymes involved in nucleotide metabolism from various *Plasmodium* species have been described (Schimandle *et al*., 1987; Walter and Königk, 1974; Lukow *et al*., 1973) and reviewed by Sherman (Sherman, 1979). One such enzyme, which is involved in the purine nucleotide salvage pathway, is hypoxanthine-guaninephosphoribosyltransferase (HGPRT; IMP: pyrophosphatephosphoribosyltransferase; EC2.4.2.8).

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HGPRT is a tetrameric enzyme, catalysing the phosphoribosylation of hypoxanthine and guanine to form inosine monophosphate (IMP) and guanosine monophosphate (GMP), in the presence of 5'-phosphoribosyl-1-pyrophosphate (PRPP) and is essential for normal purine biosynthesis in man. Partial deficiency of this enzyme leads to excessive production of uric acid and gouty arthritis, whereas a complete deficiency leads to the severe central nervous system disorder Lesch-Nyhan syndrome (Kelley and Wyngaarden, 1983). Studies of *P. falciparum*-infected red blood cells have shown that hypoxanthine is a key metabolite in parasite nucleic acid synthesis (Sherman, 1979; Königk, 1977; Yamada and Sherman, 1981; Webster and Whaun, 1981). It has also been shown that HGPRT activity is present in *P. falciparum* blood stage parasites at high levels (Reyes *et al.*, 1982). The reliance that the parasites have on their own HGPRT activity during intra-erythrocytic stages suggests the enzyme as a potential target for drug therapy in the treatment of malaria patients. The parasite enzyme has been purified from *in vivo P. falciparum* cultures and its additional specificity for xanthine is documented (Queen *et al.*, 1988; Queen *et al.*, 1989). Previous attempts to purify this enzyme *in vitro* have been unsuccessful because of the difficulty in obtaining cultured parasite material and because of the inherent instability of the enzyme during purification and storage.

Therefore, one of the possible targets for the future development of new classes of antimalarial agents, is the purine salvage enzyme hypoxanthine-xanthine-guaninephosphoribosyltransferase (HXGPRT). Moreover, the gene encoding the parasite HGXPRT has been cloned and expressed in *E. coli* (Vasanthakumar *et al.*, 1990). This gene functionally complements HGXPRT deficiency in the *E. coli* validating that a functional enzyme is produced in the strain (Shahabuddin and Scaife, 1990). However, the recombinant *P. falciparum* HGXPRT was inactive and therefore functional studies with the enzyme were not possible.

When studying mosquito-malaria interactions, model parasite systems are often used because of the ease of manipulation and the laboratory safety afforded by using parasites incapable of infecting humans. One model parasite commonly used is *P. berghei*, a rodent malaria species originally isolated from the salivary glands of *Anopheles dureni* and whose vertebrate host in nature is the Central African tree rat, *Thamnomys surdaster* (Vincke, 1954; Vincke and Lips, 1948). *P. berghei* can be genetically manipulated, and marked transgenic parasites are easily visualized in mosquito and mammalian tissues (Franke-Fayard *et al.*, 2004; Frevert *et al.*, 2005; Hillyer *et al.*, 2007; Jin *et al.*, 2007; Kingh and Hillyer, 2012).

The *P. falciparum* HGPRT is a potential drug target (Keough *et al.*, 1999; Olliaro and Yuthavong, 1999), however, the functional study is hampered by the difficulty of either culturing of *P. falciparum* (Ali, 2014) or the purification and activation challenge (Sujay Subbayya and Balaram, 2000; Subbayya and Balaram, 2002). Therefore, using rodent malaria model for investigating the HGPRT enzyme will assist in overcoming some of these problems. In the present study a simple unsophisticated method in obtaining parasite material for molecular identification and analysis of HGPRT transferase from the rodent malaria parasite *P. berghei* is described.

2. Materials and Methods

2.1. Search Plasmodia Database for *P. berghei HGPRT* Gene

The available Plasmodial databases were searched for a clone containing the full-length *P. berghei HGPRT* gene. The ESTs sequences contained in the PlasmoDB (Bahl *et al.*, 2002; Aurrecoechea *et al.*, 2009), MR4 (Wu *et al.*, 2001) and Comparasite (Watanabe *et al.*, 2007) were all explored in this procedure. Some clones were found incomplete or not contain the full-length gene. However, the Full-Mal clone “FCPb3591” from Comparasite database was the only one which contained the full-length *P. berghei HGPRT* gene. Comparasite is a database for comparative studies of transcriptomes of parasites. In this database, each data is defined by the full-length cDNAs from various apicomplexan parasites. It integrates seven individual databases, Full-Parasites, consisting of numerous full-length cDNA clones that have been produced and sequenced: 12484 cDNA sequences from *Plasmodium falciparum*, 11262 from *Plasmodium yoelii*, 9633 from *Plasmodium vivax*, 1518 from *Plasmodium berghei*, 7400 from *Toxoplasma gondii*, 5921 from *Cryptosporidium parvum* and 10966 from the tapeworm *Echinococcus multilocularis* (Watanabe *et al.*, 2007). The libraries were produced using the oligo-capping and V-capping methods. Using the constructed cDNA libraries, 5 end-one-pass nucleotide sequences were determined for a large number of these cDNAs and were mapped on the genome sequences of the parasites (Watanabe *et al.*, 2007).

2.2. Designing Oligonucleotide Primers

The PbHGPRT1F (sense) primer was designed so as to contain *Bam*HI (GGATCC restriction site at the 5’-end and the PbHGPRT1R (anti-sense) contain *Xho*I (CTCGAG) restriction site at the 5’-end. The analysis of the two primers is shown in Table 1. Using the Invitrogen–Perfect Primer and the tool Oligo Perfect Designer, the two sets of primers were made as shown in the table below. For each primer Blast (blastn) analysis against the *PbHGPRT* gene was performed

(Frevert *et al*., 2005). The primers were analyzed using the DNA Calculator from Sigma as shown in Table 1.

Table 1. Oligonucleotide primers used in this study.

Primer Name	%GC	Size	Tm(°C)	MW	Primersequence	Secondary Structure	Primer Dimer
PbHGPRT1F	34.78	23	64.54	7024.53	ATGAAAATTCCAAACAATCCTGG	weak	No
PbHGPRT1R	31.82	22	57.89	6806.36	TTAAGAAGATGACTGCATGAAT	weak	No

2.3. PCR Amplification and Gel Electrophoresis

The cDNA coding for *P. berghei HGPRT* gene was obtained as a gift from Dr. Termi Horiuchi from Dr. Sugano and Dr. Suzaki laboratory, Tokyo University, Japan. The clone: FCPb3591 of 864 bp in length contains the full length *P. berghei HGPRT* gene. The oligonucleotides primers were prepared as previously described (Gomes *et al*., 2010; Ali *et al*., 2010; Ali *et al*., 2011; Ali *et al*., 2012a; Ali *et al*., 2012b; Ali *et al*., 2013). The concentration of the clone was checked by NanoDrop-1000, and was found to be 490 ng / µl. The stock was diluted to 1/5 and used for the PCR analysis.

The PCR reaction mixture in a final volume of 25 µl contained 98 ng (1µl) of cDNA clone FCPb3591, 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 200 µM each of dNTP, primers at 0.25 mM, 1 unit of Phusion DNA polymerase (Sigma, USA) and Milli-Q water. After 5min initial denaturation at 95°C, amplification was performed using 30 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 20 sec, and extension at 73°C for 1 min in a DNA Thermal Cycler (Mj RESEARCH, PT-200, USA). The final extension was performed at 73°C for 15 min. The amplified products were analyzed by electrophoresis in 0.8% agarose in TAE gel, stained with 5 µl of ethidium bromide (10 mg / ml) and run at 100 voltage. PCR products were visualized under UV illumination and documented by photography.

3. Results and Discussion

HGPRT is a tetrameric enzyme; catalyzing the phosphorylation of hypoxanthine and guanine to form inosine monophosphate (IMP) and guanosine monophosphate (GMP), in the presence of 5'-phosphoribosyl-1-pyrophosphate (PRPP) and is essential for normal purine biosynthesis in Man (Krenitsky *et al*., 1969; Musick, 1981; Reyes *et al*., 1982; Craig and Eakin, 2000).

An approach to developing anti-malarial drugs is to use HGPRT to convert introduced purine base analogs to nucleotides that are toxic to the parasite. This strategy requires that these compounds be good substrates for the parasite enzyme but poor substrates for the human counterpart, HGPRT (Ullman and Carter, 1995). Evidence for the essentiality of HGPRT to the parasite comes from the observed antiparasitic activity of antisense oligonucleotides of HGPRT mRNA. HGPRT is also of importance to the host, with the absence and the deficiency of HGPRT manifesting as Lesch–Nyhan syndrome and gouty arthritis, respectively, so it is essential for normal purine biosynthesis in Man.

Bioinformatics tools were used to obtain the correct cDNA clone from Full–Malaria Pb5'-EST database and were used to characterize HGPRT from *P. berghei*; the rodent strain of Malaria (Altschul *et al*., 1990; Thompson *et al*., 1994). Oligonucleotide primers were designed from those data and were used in a PCR analysis to amplify HGPRT from a clone obtained by extracting the Full-Malaria database for EST sequences.

High sequence conservation among *Plasmodium* species distinguishes HGPRT. In general, conserved residues of HPRTs and bacterial XPRTs differ at positions homologous with human Leu-67 and Glu-133 (Ser-36 and Asp-88 in the XPRT of *Escherichia coli*). Solutions for the crystal structures of HPRTs reveal that the 11 conserved residues immediately flank or are very near the active site of HPRTs (Craig and Eakin, 2000).

If the crystal structures of all purine PRTs are analyzed together with the amino acid sequences reported to GenBank, there are only 2 residues (corresponding with human Gly-69 and Asp-134) that are clearly invariant. A G69E mutation virtually inactivates the human HPRT, resulting in Lesch-Nyhan syndrome, whereas a D134G mutation partially inactivates the enzyme, resulting in gouty arthritis (Sculley *et al*., 1992).

The HGPRT enzyme from *P. berghei* was analyzed and the results are presented. Figure 2 shows the sequence comparison of the HGPRT from human, rodent and primate *Plasmodium* species. Analyses of the HGPRT sequence showed that the enzyme is significantly divergent from equivalent mammalian enzymes (data not shown). There are 72 amino acid differences between PbHGPRT and PfHGPRT residues in the N-terminus of the HGPRT, far away from the active site. This could modulate substrate binding by altered packing in the protein (Lee *et al*., 1998). Notably, *P. berghei*

HGPRT has two amino acid substitutions; where it has T-32 instead of I-32 and D-158 instead of E-158. Table 2 shows the identity percentage between HGPRT from human, rodent and primate malaria parasites. High identity in HGPRT is observed between the two rodent malaria parasites (94.81%), between *P. falciparum* and *P. reichenowi* (99.57%) and between *P. vivax* and *P. knowlesi* (92.70%).

The FCPb3591 cDNA clone was found to contain the full length gene and the obtained band size of 0.69 kb as shown in Figure 1, is in consistent with the predicted size from the nucleotides sequence contained in the clone. The *HGPRT* gene was successfully amplified by PCR from cDNA clone of *P. berghei*.

Progress in enzyme structure-based inhibitor design/discovery shows promise that HPRT inhibitors eventually might be developed into drugs for the treatment of diseases caused by parasites. For example, Hadacidin, an inhibitor of the parasite adenylosuccinate synthetase exhibits antiparasitic activity, making this pathway a target for the design of antimalarials (Webster *et al*., 1984). Formation of GMP from IMP involves two enzymes inosine monophosphate dehydrogenase (IMPDH) and guanosine monophosphate synthase. Bredinin, an inhibitor of IMPDH exhibits antiparasitic activity (Webster and Whaun, 1982). Both these purine pathways, important to parasitized erythrocytes offer potential targets for antimalarial chemotherapy.

Table 2. Sequence identity (%) between HGPRTases of human, rodent and primate malaria parasites.

% Identity	Pb HGPRT	Pf HGPRT	Pk HGPRT	Pr HGPRT	Pv HGPRT	Py HGPRT
PbHGPRT	-	67.53	67.97	67.53	67.53	94.81
PfHGPRT	67.53	-	78.35	99.57	79.22	67.97
PkHGPRT	67.97	78.35	-	78.35	92.70	67.97
PrHGPRT	67.53	99.57	78.35	-	79.22	67.97
PvHGPRT	67.53	79.22	92.70	79.22	-	68.40
PyHGPRT	94.81	67.97	67.97	67.97	68.40	-

The percent Identity Matrix created by Clustal 2.1. PbHGPRT is *P. berghei* HGPRT, PfHGPRT is *P. falciparum* HGPRT, PkHGPRT is *P. knowlesi* HGPRT, PrHGPRT is *P. reichenowi*, PvHGPRT is *P. vivax* HGPRT and PyHGPRT is *P. yoelii* HGPRT.

Several studies have investigated the metabolic enzymes HGPRT and designed some chemical compounds and derivatives that can act as anti-parasitic drugs (Baszczyňski *et al*., 2013; Krečmerová *et al*., 2012; Keough *et al*., 2009).

Comparative analysis of the HGPRT enzymes from Plasmodia may give new inputs as to which groups of the Plasmodia HGPRTases are suitable for functional investigation as anti-malarial drug target.

Work presented in this paper will be useful in producing recombinant protein for structural and functional studies. Because of the difficulty of the purification of other human plasmodial HGPRT, results of this study can assist in the investigation of HGPRT as one of the possible targets for the future development of new classes of anti-malarial agents.

In conclusions, this study demonstrates bioinformatics and genetic investigations of metabolic enzyme of Plasmodia parasite; the HGPRT, as a step for verification its potentiality as a drug target.

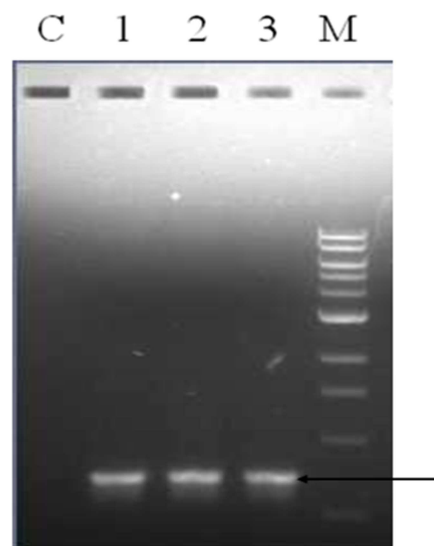

Figure 1. Polymerase Chain Reaction (PCR) Identification of the cDNA clone of the P .berghei HGPRT.

A 696 bp specific fragment was amplified from the cDNA of *P. berghei* using a pair of specific primers designed according to the assembled HGPRT cDNA sequence. M; DNA marker. Lanes 1, 2 and 3; 49 ng, 98 ng and 198 ng of cDNA, respectively. 2 µl of PCR reaction were loaded in wells 1–4. PCR product of HGPRT cDNA fragment is indicated by arrow. C is negative control without adding DNA.

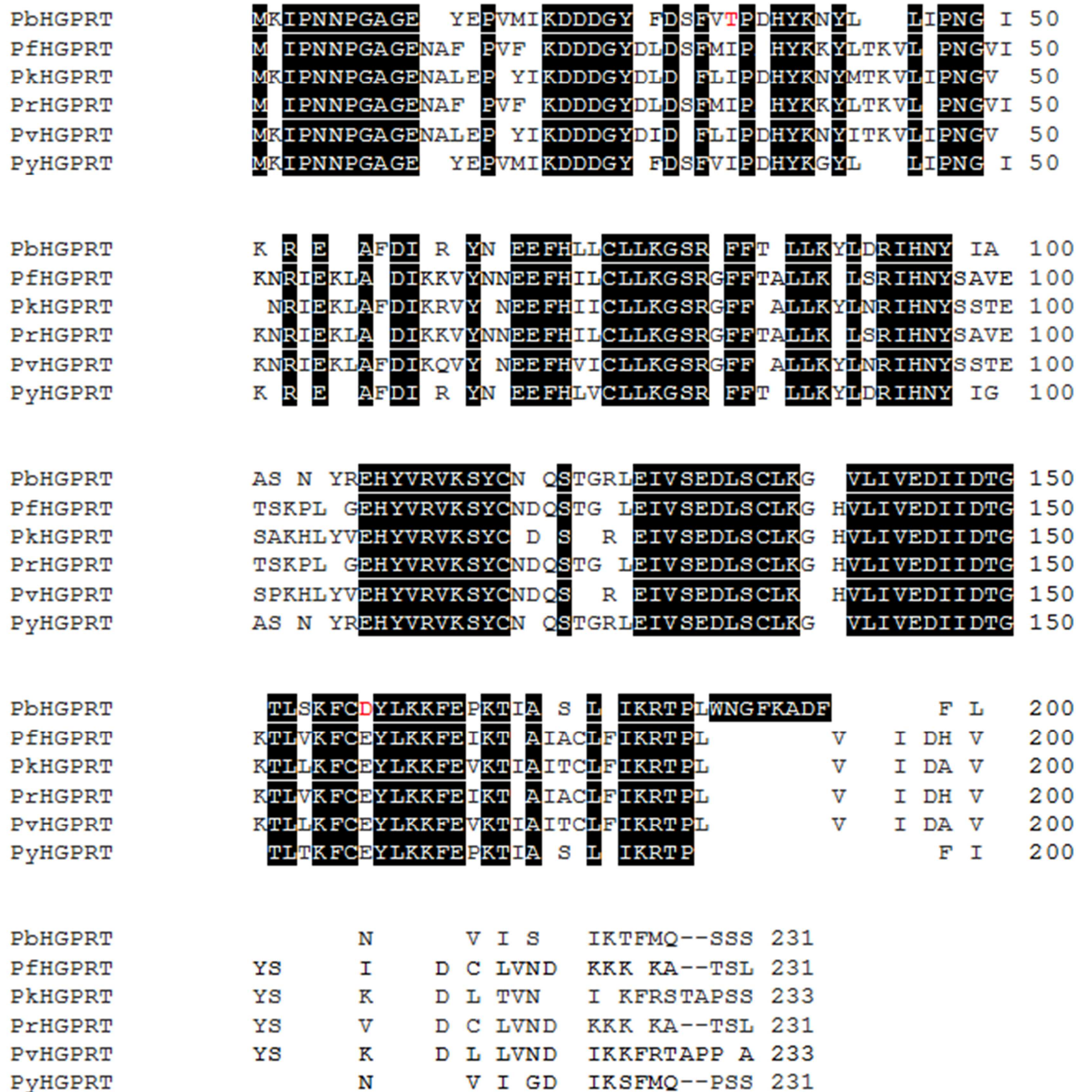

Figure 2. Comparison of the Amino Acid Sequence of *P. berghei* HGPRT (PbHGPRT) with *P. falciparum* HGPRT (PfHGPRT), *P. knowlesi* HGPRT (PkHGPRT), *P. reichenowi* (PrHGPRT), *P. vivax* HGPRT (PvHGPRT) and *P. yoelii* HGPRT (PyHGPRT). Dashes indicate gaps made to maximize alignments. The underlined residues represent the active site. The black shading represents residues those are identical in all sequences, grey shading represents residues those are identical but with one or two difference in the six HGPRTs.

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