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Research note

Molecular cloning of a gene encoding a 20S proteasome β subunit from *Plasmodium falciparum*☆

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Abstract

A novel gene was cloned from *Plasmodium falciparum*. Database searches indicated this gene to be a member of the 20S proteasome β-subunit family. Comparison of the gene's genomic DNA sequence with cDNA sequence revealed a 156-bp intron 85 bp downstream from the start codon. The nucleotide sequence of the gene contains one open reading frame encoding 265 amino acids with a predicted molecular mass of 30.9 kDa and a p*I* of 6.2. Northern blot analysis showed the transcript size to be approximately 1.6 kb indicating that some 800 bp of the transcript is non-coding.

Keywords: Beta-subunit; Multicatalytic protease complex; *Plasmodium falciparum*; Proteasome

Proteasomes are multicatalytic ATP-dependent protease complexes. They play a critical role in a number of biological processes by means of selective protein degradation in both the cytosol and the nucleus [1,2]. The hollow "cylinder-like" eukaryotic 20S proteasome consists of four stacked rings. The outer rings are made up of seven different alpha-subunits and the inner ring is composed of seven different beta-subunits. The 20S proteasome works as a functional core and forms the enzymaticaly active 26S proteasome when attached to two terminal regulatory subcomplexes [3–5].

Genes encoding alpha- and beta-subunits of the 20S proteasome have been cloned from many organisms including archaebacteria, yeast, Drosophila, Xenopus, rat and human [6–10]. It is assumed that the malaria parasite *P. falciparum* has a functional proteasome. A recent biochemical study has identified the parasite proteasome as a potential chemotherapeutic target [11] although there is no published molecular evidence to confirm the presence of this complex in the malarial parasite. Here we report the complete sequence of a novel gene encoding a *P. falciparum* 20S proteasome beta-subunit (PfPB).

Arbitrary-priming PCR or RT-PCR has been widely used in genome fingerprinting, genetic polymorphisms and mRNA differential display [12–14]. As part of a study aimed at identifying genes involved in drug resistance in *P. falciparum* we have used arbitrary-priming RT-PCR to examine mRNA polymorphisms in a pair of genetically related isolates one of which was chloroquine-resistant (K1 strain) and the other chloroquine-sensitive (K1HF) [15]. Total RNA from the two

Abbreviations: PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR.

☆ Note: Nucleotide sequence data reported in this paper is available in the EMBL, GenBank® and DDJB databases under the accession number AF090371.[1]

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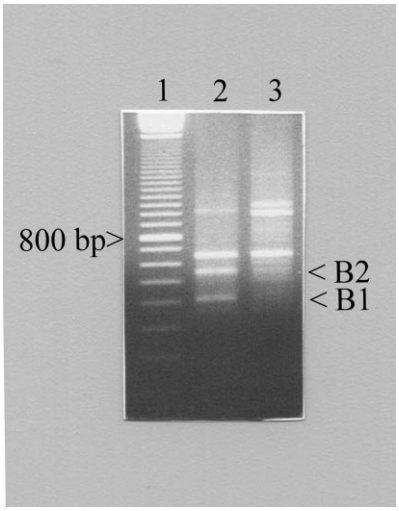

Fig. 1. Arbitrary-priming RT-PCR results. Lanes 1 = 100 Base-pair ldder; 2 = K1-strain PCR product; 3 = K1HF-strain PCR product. Arrows indicate the bands of interest.

strains was isolated with TRIZOL Reagent (GIBCO BRL). After DNase treatment, the RNAs were used in first-strand cDNA synthesis with T-Primed First-Strand Kit (Pharmacia Biotech). The Not I-d$(T)_{18}$ primer (5′-AACTGGAAGAATTCGCGGCCGCAG-$GAAT_{18}$-3′) in the First-Strand Reaction Mix was in sufficient quantity to prime cDNA synthesis and to serve as a downstream PCR primer. An arbitrary primer (5′-GAATTCGCGGCCGCAGGAAT-3′) was used as the upstream primer. After an initial 3 min denaturation step at 94°C, 40 PCR cycles were carried out, each consisting of a 30 s denaturation at 94°C, 30 s annealling at 60°C and 1 min extension at 72°C. Agarose-gel electrophoresis of the RT-PCR products indicated two bands of interest. Bands of 410 bp (B1) and 550 bp (B2) were in much greater abundance in the K1 than the K1HF isolate (Fig. 1).

The B2 fragment (RT-F) was cloned into pGEM-T vector (Promega) and subjected to sequencing. The RT-F nucleotide sequence contained an open reading frame (ORF) and a stop codon near to its 3′ end but no start condon. Blast searching against protein databases indicated that the fragment encoded part of a 20S proteasome beta-subunit gene (PfPB). To obtain the whole PfPB sequence three upstream primers (5′-TGTTTTTATATTCATTCTGTTCATCATCA-3′, 5′-TATTGAGCATCAGCTAGCTCACCA-3′, and 5′-GTTACCTTTTCTATCTGCTGCAATCA-3′) and one downstream primer (5′-CCTGCTGGATG-TATGTGGTGATTG-3′) were designed based on the newly obtained sequences. These were used to screen Vectorette libraries constructed by ligation of an oligonucleotide link (named vectorette) to restriction-enzyme digested genomic DNA fragments from the 3D7 strain of *P. falciparum,* such that a universal primer compatible with the vectorette could be used in PCR together with the specific primer [16]. This strategy produced three new upstream PCR fragments which were from TaqI digested DNA (Taq I–1 frag-

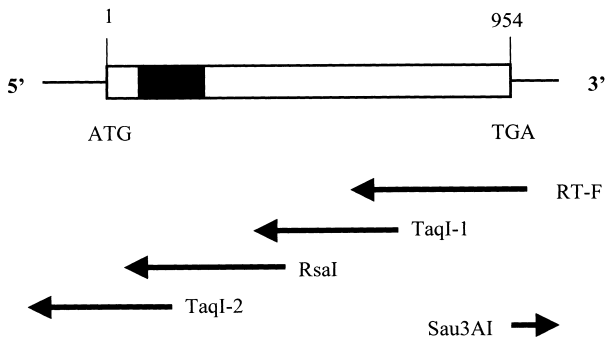

Fig. 2. The cloning strategy scheme. Numbers indicate the start and stop nucleotide of ORF (open box) of PfPB. The solid box indicate the intron region. Arrows indicate cloning fragments

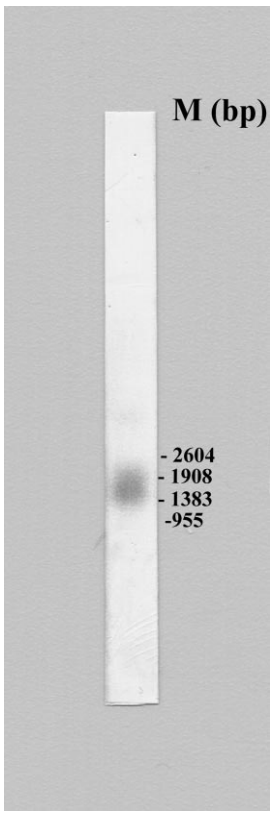

Fig. 3. Northern blot analysis of the PfPB gene. The total RNA of *P. falciparum* 3D7 strain was electrophoresed on 1% agarose gel under denaturing conditions using formamide and formaldehyde in MOPS buffer and transferred onto a nylon membrane (Hybond-N). The membrane was hybridised with random primed probe, corresponding to the whole encoding region of PfPB, at 42°C overnight in a solution containing 50% formamide, 6 × SSC, 5 × Denhardt's solution, 0.5% SDS and 100μg ml^{-1} salmon sperm DNA. Washes were carried out with 2 × SSC, 0.5% SDS for 10 min at room temperature, 1 × SSC, 0.1% SDS for 30 min at 42°C and 0.1 × SSC, 0.1% SDS for 30 min at 65°C.

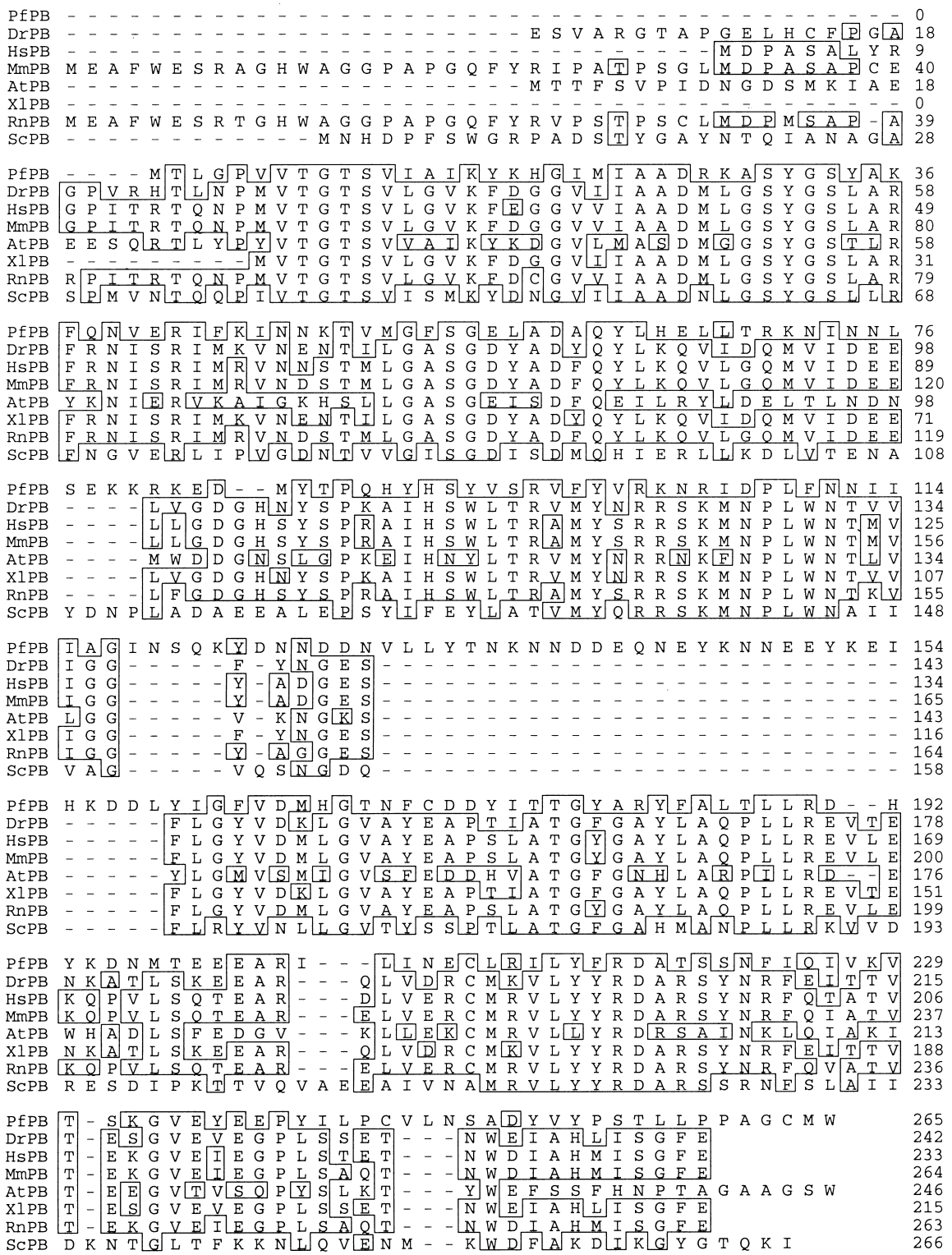

Fig. 4. Alignments of the PfPB amino acid sequence with other 20S β-subunit proteasome homologues from other species were performed using LASERGENE software for windows. Boxed residues are identical with at least three sequences. DrPB = proteasome beta chain precursor from zebrafish *Danio rerio* (P28024); HsPB = proteasome beta-subunit from human (S45719); MmPB = proteasome beta chain precursor from mouse *Mus musculus* (P99026); AtPB = proteasome beta subunit from *Arabidopsis thaliana* (AF043538); XlPB = proteasome from African clawed frog *Xenopus laevis* (S17568); RnPB = proteasome beta chain from Norway rat *Rattus norvegicus* (P34067); ScPB = proteasome component from yeast *Saccharomyces cerevisiae* (P30657).

ment), RsaI digested DNA (RsaI fragment) and another TaqI digested DNA (TaqI–2 fragment), respectively, and one downstream PCR fragment which was from Sau3AI digested DNA (Sau3AI fragment). Sequencing results identified overlapping regions between adjacent fragments and a start codon was identified in TaqI–2. The complete coding region of the PfPB was obtained from the four Vectorette PCR fragments and the RT-F fragment (Fig. 2). The four vectorette PCR fragments were obtained from the 3D7 strain while the RT-F fragment was from the K1 strain. Therefore, two specific primers flanking the RT-F sequence were used to obtain the same fragment from 3D7 strain gDNA by PCR. The sequencing result indicated that the fragments from the K1 and 3D7 strains were identical.

Previous studies revealed that 20S proteasome subunit genes contain at least one intron [17,18]. In order to check for introns in the *P. falciparum* proteasome and to cross-check the correctness of the genomic DNA sequence alignment, the complete cDNA sequences of 3D7, HB3, K1, and K1HF strains were obtained by sequencing RT-PCR products produced using two primers (5′-GTAATATTAAA AAGGAAA-GAAAGAAA-3′ and 5′-AATAACTTCTTGGGATA-CAGCG-3′) flanking the start and stop codons. Comparison of the cDNA sequence with the genomic DNA sequence indicated the absence of a 156-bp long sequence 85 bp downstream from the start codon in the cDNA sequence. The missing 156-bp fragment had a typical splice consensus sequence with GT at its 5′ end and AG at its 3′ end confirming the presence of a 156-bp intron within the coding region (Fig. 2). cDNA sequencing results confirmed the correctness of the genomic DNA sequence alignment and there were no differences at the cDNA level between the four strains studied. Northern blot analysis of total malaria parasite RNA identified the transcript of the PfPB to be approximately 1.6 kb (Fig. 3) indicating that some 800 bp of the transcript is non-coding, a common characteristic of *P. falciparum* genes [16,19].

The PfPB nucleotide sequence contains one ORF which is interrupted by a 156-bp intron and encodes 265 amino acids with a predicted M_r of 30.9 kDa and a pI of 6.2 (obtained through LASERGENE software for windows). The M_r is within the 21–32 kDa M_r range which is typical for eukaryotic proteasome subunits [20]. The likely start codon of ATG was based on the following reasons: The A/T content of the sequence increases markedly upstream from this codon as is seen in many other *P. falciparum* genes [21]; there are two in-frame stop codons upstream from the codon, located at −78 and −162, respectively; the sequence flanking the start codon is AAAA/ATG which is commonly seen in *P. falciparum* genes [19,22,23].

Using the complete nucleotide sequence of PfPB Blast searches in GenBank databases were carried out. Results indicated that PfPB is a member of the 20S proteasome beta-subunit family. The highest homology with PfPB was to a fish (*Danio rerio*) proteasome beta-subunit with 65.3% similarity and 38.5% identity. Protein sequence alignments from the seven top-scoring genes as identified by Blast Search are shown in Fig. 4. The whole sequence is similar to those of 20S proteasome beta-subunits originating from seven different living organisms except they contained an insert of 30 amino acid residues. It is not clear whether the insert is a unique feature in 20S proteasome beta-subunits of malaria parasites. Since no enough data are available at present it is also difficult to define the subunit to any specific group beta-subunits.

One recent study has shown that overexpression of a human 26S proteasome subunit is associated with pleiotropic drug resistance in fission yeast. When the gene was transiently overexpressed in mammalian cells it conferred P-glycoprotein-independent resistance to taxol, doxorubicin, 7-hydroxystaurosporine and ultraviolet light [24]. The RT-PCR results shown in Fig. 1 suggest differences in transcript number between the K1 and K1HF isolates. We propose to determine if this results in overexpression of the β-subunit in the K1 isolate which could play some role in drug resistance.

Acknowledgements

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