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Identification and function of a shrimp white spot syndrome virus (WSSV) gene that encodes a dUTPase

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Abstract

The ORF *wsv112* of shrimp white spot syndrome virus (WSSV) was predicted to encode a protein with five conserved motifs at its N-terminus characteristics of dUTPases. The transcription of the gene named as *wdut* was analyzed by RT-PCR and RACE. The C-terminal end of the putative WSSV dUTPase bore very low similarity to the reported dUTPases and any other known proteins. Therefore, the 5′-terminal region (528-bp) of *wdut* gene was expressed in *E. coli*. The recombinant WSSV dUTPase (WDUT) with a molecular mass of 23 kDa could catalyze the hydrolysis of dUTP into dUMP and was highly specific for dUTP with an apparent Km of 1.2 μM. Furthermore, gel filtration results revealed that this enzyme was a trimer.

Keywords: White spot syndrome virus; WSSV; dUTPase

1. Introduction

White spot syndrome virus (WSSV) is the causative agent of WSS that is highly virulent in shrimp, the most important species used in aquaculture, and can also infect most species of crustacean (Lo et al., 1996; Chen et al., 2000; Hameed et al., 2003). In previous studies, the complete genome sequence of WSSV has been determined (Yang et al., 2001; van Hulten et al., 2001b), but most of the putative ORFs encode proteins bearing no homology to any known proteins or motifs. So far, only some major structural proteins (Van Hulten et al., 2001a; Huang et al., 2002; Zhang et al., 2002) and a few functional proteins have been identified. Genomic sequence analysis (Yang et al., 2001) showed that WSSV may encode enzymes involved in nuleotide metabolism, including a dUTPase (ORF *wsv112*). These enzymes also include thymidine kinase (TK) (Tzeng et al., 2002), ribonucleotide reductase (RR) (Lin et al., 2002) and thymidylate synthase (TS) (Li et al., 2004), the only three WSSV enzymes experimentally proved to be functionally active. Therefore, further studies on other unknown proteins are necessary to advance our understanding of this distinct virus at the molecular level.

dUTP pyrophosphatase (dUTPase; EC3.6.1.23) plays an essential role in nucleotide biosynthesis. Hydrolysis of dUTP by dUTPase produces dUMP, required for the de novo synthesis of dTTP, and maintains low cellular ratios of dUTP:dTTP, thus preventing the misincorporation of uracil into chromosomal DNA (Kornberg and Baker, 1991; Tye et al., 1977). The gene encoding dUTPase (*dut*) is present in most organisms from higher eukaryotes to viruses. Several studies indicate that the expression of cellular dUTPase is cell-cycle dependent and/or developmentally regulated (Pardo and Gutierrez, 1990; McIntosh et al., 1994; Strahler et al., 1993). The presence of dUTPase in several virus families, such as retroviruses, herpesviruses and poxviruses (Elder et al., 1992; Wohlrab and Francke, 1980; Broyles, 1993), suggests that the strict control of dUTP levels is critical in the replication of many kinds of viruses (Pyles and Thompson, 1994; Turelli et al., 1996; Oliveros et al., 1999). Some viruses encode dUTPase to be free from normal cellular regulatory constraints and to prevent the synthesis of mutagenic uracil-

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substituted DNA. African swine fever virus (ASFV) dUTPase activity is dispensable for virus replication in dividing cells but is required for productive infection in nondividing swine macrophages, the natural host cell for the virus (Oliveros et al., 1999). The viral dUTPase may play a role in minimizing misincorporation of uracil into the viral DNA and ensure the fidelity of genome replication.

In this paper we cloned the WSSV dUTPase gene (*wdut*) and characterized the recombinant enzyme as a homotrimeric dUTPase that showed a high specificity for dUTP.

2. Materials and methods

2.1. Amino acid sequence comparisons and phylogenetic analysis

The Blast search facility in the GenBank database was used for homology analysis of the ORF *wsv112*. Multiple alignment and phylogenetic analyses were performed using the DNAMAN software (Lynnon Biosoft, Vaudreuil, Canada). One hundred bootstrap replicates were generated to test the robustness of the trees. Fifty-two selected dUTPases (Table 1) available in GenBank were used in the alignment and phylogenetic analyses.

2.2. RNA preparation

The RNAs for the transcriptional analysis were extracted from WSSV-infected crayfish at different times postinfection. The viral inoculums used for injection were extracted from diseased *Penaeus Japonicus*, which were collected from Xiamen, China. Tissues (muscle) were homogenized in 0.9% NaCl at 0.1 g/ml. After centrifugation at 3000 × *g* for 10 min, the supernatant was filtered (0.45 μm filter) and injected intramuscularly into crayfish *Cambarus clarkii* (collected from Anhui province, China) in the lateral area of the fourth abdominal segment. At various infectious stages (i.e. 0, 2, 4, 6, 8, 12, 18, 24 and 48 h postinfection, h.p.i.), three specimens were selected at random and their hepatopancreas were excised. The collected hepatopancreas was immediately used for isolation of total RNA using the SV Total RNA Isolation System (Promega). Total RNA was quantified by spectrophotometry at 260 nm.

2.3. Temporal analysis of wdut transcription by RT-PCR

Total RNA (0.5 μg) was denatured by heating at 70 °C for 5 min in 10 μl DEPC-water containing 200 ng oligo dT primer (5′-TTTTTTTTTTTTTTTT-3′), then chilled on ice for 5 min. The first-strand cDNA was synthesized by the addition of 200 U M-MLV reverse transcriptase (Promega), 4 μl M-MLV reverse transcriptase 5× reaction buffer, 2 μl 10 mM dNTPs and 20 U RNasin (Promega). DEPC-water was added to make a final volume of 20 μl. The reverse transcription proceeded at 42 °C for 1 h, followed by heating at 70 °C for 15 min to stop the reaction. Two microliters of the products of the cDNA reaction was subjected to PCR in a 50-μl reaction buffer containing 100 pmol of each primer (P1/5SP for *wdut*: 5′-GAGAGGATCCGACTCATCTGCATCTGTCGTG-3′ / 5′-AATGCTGGTTCCCTTTC-3′). The PCR cycles were: 94 °C for 5 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, followed by an elongation at 72 °C for 5 min. The transcription of actin with actin1/actin2 primer set (5′-TTTCGCCCTTCCTCATGCCA-3′/5′-TGCCAGCGCAGT GATTTCCT-3′) served as an internal control for RNA quality and amplification efficiency. At the same time, 0.5 μg of total RNA was treated with 10 U RNase free of DNase (Promega), and subjected to RT-PCR as described above. This negative control was used to confirm that the RNA was not contaminated by viral DNA.

2.4. Determination of the 5′- and 3′-terminal region of the wdut transcript

Based on the nucleotide sequence of *wsv112*, the 5′ and 3′ ends of the cDNA encoding WSSV dUTPase were obtained by 5′ and 3′ RACE using a commercial 5′/3′ RACE kit (Roche), according to the manufacturer's recommendations. The RNA samples used in this study were extracted from WSSV-infected crayfish 24 h.p.i. and then treated with RNase-free DNase. For 5′ RACE, the first-strand cDNA was synthesized using the random hexa-oligo primer and a poly(A) tail was added to the cDNA products using terminal transferase in the presence of dATP. The gene-specific primer P2 (5′-GAGCGAGCTCTTCAGTAAAATTTGGGTT-3′) and oligo(dT)-anchor primer (5′-GACCACGCGTATCGATG TCGACTTTTTTTTTTTTTTTA/G/C-3′) supplied in the kit were used for PCR. The PCR product after the first cycle was used as the template for the second amplification using gene-specific primer 5SP (5′-AATGCTGGTTCCCTTTC-3′) and anchor primer (5′-GACCACGCGTATCGATGTCGAC-3′). For 3′ RACE, first-strand cDNA was synthesized using oligo(dT)-anchor primer. The gene-specific primer 3SP (5′-TACGAACACTACGACGC-3′) and the anchor primer supplied with the kit were used for PCR. The PCR products from 5′ and 3′ RACE were purified on a 1.5% agarose gel and subcloned into the pMD18-T vector (TaKaRa), respectively. Arbitrarily selected clones were sequenced and compared with the genomic DNA sequence of WSSV.

2.5. Cloning of wdut

The DNA fragment corresponding to the N-terminal 176 amino acid residues of putative WSSV dUTPase was amplified from the intact WSSV genomic DNA with the primer P1 (5′-GAGAGGATCCGACTCATCTGCATCTGTCGTG-3′) and P2 (5′-GAGCGAGCTCTTCAGTAAAATTTGGGTT-3′). *Bam*HI and *Sac*I sites present in primer P1 and primer P2, respectively, were used for cloning *wdut* into vector pQE30