Identification of the thymidylate synthase within the genome of white spot syndrome virus

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Thymidylate synthase (TS) (EC 2.1.1.45) is essential for the de novo synthesis of dTMP in prokaryotic and eukaryotic organisms. Within the white spot syndrome virus (WSSV) genome, an open reading frame (WSV067) that encodes a 289 amino acid polypeptide showed significant homology to all known TSs from species including mammals, plants, fungi, protozoa, bacteria and DNA viruses. In this study, WSV067 was expressed in Escherichia coli, and the purified recombinant protein showed TS activity in dUMP–folate-binding assays using ultraviolet difference spectroscopy. RT-PCR and Western blot analyses showed that WSV067 was a genuine and early gene. Phylogenetic analysis revealed that WSSV-TS was more closely related to the TSs of eukaryotes than to those from prokaryotes.

INTRODUCTION

White spot syndrome virus (WSSV) is a major pathogen that is mainly found in the cultivated shrimp. Although the determination of the WSSV genomic DNA sequence from three different isolates (GenBank accession nos AF332093, AF369029 and AF440570) has greatly facilitated the investigation of pathogenicity-related genes or gene products of WSSV, and some major structural proteins (Van Hulten et al., 2001; Huang et al., 2002; Zhang et al., 2002) and enzymes (Tsai et al., 2000; Van Hulten et al., 2000; Chen et al., 2002) have been identified, the interaction between WSSV and its host has still not been well defined. Thus there is an urgent need to study further virus gene products to clarify the infectious mechanism and discover potential therapeutic targets or methods for prevention and treatment of this disease.

Within the genome of WSSV, on the basis of the presence of highly conserved motifs, WSV067 encoding a putative thymidylate synthase (termed WSSV-TS) has been tentatively characterized (Yang et al., 2001). It is located at position 31092–31958 bp in the genome and encodes a 289 aa protein (32·6 kDa).

Thymidylate synthase (TS) is essential for the de novo synthesis of deoxythymidine monophosphate (dTMP) in prokaryotic and eukaryotic organisms by catalysing the reductive methylation of 2’-deoxyuridylate (dUMP) by 5,10-methylenepterahydrofolate to give dTMP and dihydrofolate. Consequently it plays a major role in the DNA replication of a cell or a DNA virus (Perryman et al., 1986) and has been used successfully as a therapeutic target for the treatment of proliferation diseases such as cancer (Danenberg, 1977).

The catalytic mechanism of TS has been widely studied in the past, and much is known about the structure and function of the enzyme (Perry et al., 1990). In protozoans and plants, TS combines with dihydrofolate reductase (EC 1.5.1.3) to form a bifunctional dihydrofolate reductase–TS (DHFR–TS). For DNA viruses, TS is only found in bacteriophage (Belfort et al., 1983b; Kenny et al., 1985), herpesvirus (Bodemer et al., 1986; Richter et al., 1988; Russo et al., 1996) and three insect viruses including Chilo iridescent virus (Invertebrate iridescent virus 6; IRV6) (Muller et al., 1998), Melanoplus sanguinipes entomopoxvirus (MSEV) (Afonso et al., 1999) and Heliothis zea virus 1 (HzV-1) (Chen et al., 2001).

In this work, homologous and phylogenetic analyses were performed to study the evolutionary relationship of WSSV-TS using known TS sequences in the SWISS-PROT database. The transcription and expression of WSSV-ts was identified with RT-PCR, rapid amplification of cDNA ends (RACE) and Western blot analyses. The WSSV recombinant TS protein (termed rTS) was expressed in Escherichia coli and was functionally identified by dUMP–folate-binding activity assay.

METHODS

Crayfish Cambarus clarkii infection with WSSV. The virus inoculum used for injections was extracted from diseased Penaeus japonicus showing the prominent characteristic white spots, and which were collected from Xiamen, China. Tissues (muscle, heart
and gill) were homogenized in TNE buffer (20 mM Tris/HCl, 400 mM NaCl, 10 mM EDTA, pH 7.4) at 0-1 g ml⁻¹. After centrifugation at 3000 g for 10 min, the supernatant was filtered (0-45 μm filter) and injected intramuscularly into C. clarkii (collected from Anhui Province, China) in the lateral area of the fourth abdominal segment. At various stages [i.e. 0, 2, 4, 6, 12, 24, 48 and 72 h post-infection (h p.i.)], the hepatopancreas was freshly avulsed from the infected crayfish, frozen immediately in liquid nitrogen and kept there until use.

**Homologous and phylogenetic analyses of WSSV-TS.** The amino acid sequences of TS from mammals, fungi, bacteria, protozoa and DNA viruses in the SWISS-PROT databases were used in homologous and phylogenetic analyses. The homologous analysis was performed using dNAMAN software (Lynnnon BioSoft). Amino acid sequences of TS from human (TYSY_HUMAN), mouse (TYSY_MOUSE), rat (TYSY_RAT), human herpesvirus 8 (TYSY_KSHV), herpesvirus saimiri (TYSY_HSVSA), equine herpesvirus type 2 (TYSY_SHVE2), herpesvirus ates (TYSY_SHVAT), varicella-zoster virus (TYSY_VZVD), fruitfly (TYSY_DROME), mushroom (TYSY_AGABI), baker’s yeast (TYSY YEASE), HIV-1 (TYSY_HIV1), IRV6 (TYSY_IRV6), MERV (TYSY_MSEV), Escherichia coli (TYSY ECOLI), bacteriophage T4 (TYSY BPT4), Crithidia fasciculata (DRTS CRIFA), carrot (DRTS DAUC) and soybean (DRTS SOYBN) were used in the multiple sequence alignment (Table 1).

Phylogenetic analysis was performed using dNAMAN to produce input files of aligned protein sequences. A phylogenetic tree was drawn to investigate the evolutionary position of WSSV-TS.

**Transcriptional analysis of gene.** Total RNAs, extracted from the hepatopancreas of WSSV-infected crayfish at different times after infection (i.e. 0 to 72 h p.i.), were treated with DNase and reverse-transcribed. The cDNAs were subjected to PCR using ts-specific forward and reverse primers (5′-Tcaacctcatacaatag-3′, 5′-caggataaccatcattgtc-3′). The PCR cycles were as follows: 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min, followed by an elongation at 72 °C for 10 min. The cDNA products were used as the internal control for RT-PCR with a gene-specific forward and reverse primers (5′-CTGAGTTGTCGTGGTGTGTG-3′ and 5′-TTCAGTGCATCTTCTCAC-3′). Total RNA from healthy crayfish was cloned into the pQE30 vector (Qiagen). The recombinant plasmid was transformed into E. coli BL21 (DE3) cells. Liquid cultures were grown in a shaking incubator (200 r.p.m.) at 37 °C until the OD₆₀₀ reached 0-5 and these were then induced with 0-5 mM IPTG for 8 h at 28 °C. The cells were harvested by centrifugation at 4000 g for 5 min. The recombinant WSSV-TS (termed rTS) was purified by Ni-NTA affinity chromatography under native conditions following methodology in the QiAexpressistion handbook (Qiagen). The E. coli cells containing pQE30 vector were also induced with IPTG and total protein extracts were applied to the Ni-NTA column as described above. Final eluates were collected and used as the negative control (termed NC).

**Preparation of antibody.** The purified rTS was used as an antigen to immunize mice by intradermal injection once every 10 days. Antigen (100 μg) was mixed with an equal volume of Freund’s complete adjuvant (Sigma) for the first injection. The subsequent three injections were conducted using 100 μg antigen mixed with an equal volume of Freund’s incomplete adjuvant. Four days after the last injection, mice were exsanguinated and the antisera was collected. The antiserum titer is determined by ELISA using horseradish peroxidase-conjugated goat anti-mouse IgG (Promega). For a negative control, antigen was replaced with 1 × PBS.

**Western blot.** Total proteins, extracted from hepatopancreas of infected crayfish at various times (i.e. 0, 2, 4, 6, 12, 24, 48 and 72 h p.i.), were separated by SDS-PAGE. These proteins were transferred onto a PVDF membrane (Amersham Pharmacia). The membrane was then immersed in blocking buffer (2% BSA, 20 mM Tris, 150 mM NaCl, 0-1% Tween 20, pH 7-5) at room temperature for 30 min, followed by incubation with anti-rTS serum (diluted 1:2000) for 1 h. Following this, alkaline phosphatase-conjugated goat anti-mouse IgG (Promega) was used as the secondary antibody. Detection was performed using Western Blue Stabilized Substrate for Alkaline Phosphatase (Promega).

**UV difference spectroscopy analysis.** This was performed as described (Lockshin et al., 1984). In brief, 5 μl of 0-424 mM dUMP was added to 500 μl buffer (50 mM TES pH 7-4, 25 mM MgCl₂, 1-0 mM EDTA, 5-0 mM DTT) containing 5 μl purified rTS and 10 μl of 3-6-μM folate (Sigma). The reaction was conducted in a sample cuvette at room temperature in a spectrophotometer thermostatic chamber. The absorbance was recorded within the 250–360 nm range and the final difference spectra, representing the rTS-dUMP–folate complex, were obtained. In the negative control, the same dUMP dose was added to the sample cuvette containing the same buffer but substituting the NC fraction mentioned above.

**RESULTS**

**Multiple sequence alignment and evolutionary analysis of WSSV-TS**

Amino acid sequences of 20 TS or DHFR–TS from mammals, yeasts, bacteria, DNA viruses, protozoa and plants were used for the multiple sequence alignment. The results showed that there was significant homology with five conserved motifs including the folate-binding site (Chiericatti & Santi, 1998) (Fig. 1, motif 1), catalytic centre region (Carreras & Santi, 1995) (Fig. 1, motif 2), dUMP-binding site (Tong, 1998) (Fig. 1, motif 3) and proton transport region (Carreras & Santi, 1995) (Fig. 1, motif 4). These motifs are essential for the biofunction of TS (Carreras & Santi, 1995). The consensus sequence GDLGPVYGFQWRHFA (Fig. 1, motif 5) was highly homologous.
Table 1. Information on TS sequences compared

Forty-two TS amino acid sequences, from prokaryotic and eukaryotic organisms including mammals, fungi, bacteria, protozoa and DNA viruses, were used in homologous and phylogenetic analyses. The accession numbers of each of the TS sequences and their similarity to WSSV-TS are also indicated in the table.

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<th>TS</th>
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<th>Organism</th>
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<th>Reference</th>
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conserved in almost all the TS, but its function is currently unknown.

To investigate the evolutionary relationship of WSSV-TS, phylogenetic analysis was performed using all known TSs, including TS and DHFR–TS, in the SWISS-PROT database. Forty-two TSs from representative organisms of different species, including prokaryotes and eukaryotes, were selected to draw the phylogenetic trees using DNAMAN (Table 1). The homology matrix analyses revealed that WSSV-TS had
the highest similarity to DRTS_TRYBB (64.2%) and TYSY_HUMAN (63.6%) and the lowest similarity to TYSY_BACMO (34.3%) and TYSY_BPPHT (33.9%) (see Table 1). The similarity of WSSV-TS to TS from eukaryotes (50.2–64.2%) was higher than those from prokaryotes (33.9–49.2%). Its phylogenetic position indicated that WSSV-TS had the closest evolutionary relationship to the TS from parasitic protozoa of insects, and the furthest relationship to TS from bacteria and bacteriophage (Fig. 2), which was identical to what was found for the homology analysis results.

Mapping of the 5’ and 3’ ends of the WSSV-TS transcripts

The 5’-RACE products formed a single 150 bp band (Fig. 3a). Sequencing analysis of the products revealed that the 5’ terminus was located 22 nt upstream of the predicted initiation codon (Fig. 3c), and a putative TATA box was found at 28 nt upstream of the transcriptional initiation sites. The sequence either side of the putative translation initiation codon (AATATGG) complied with the Kozak rule (Kozak, 1989).
The 3'-RACE fragments (Fig. 3b) were also cloned and sequenced. Although there was no typical polyadenylation signal (AATAAA), a poly(A) tail was added 116 nt downstream of the termination codon. This result indicated that other undefined signal pathways that regulate WSSV-ts polyadenylation may exist. The consensus sequence CGTGTTAG, which was identical to the mRNA polyadenylation-related signal (PyGTGTTPyPy) of herpesvirus (McLauchlan et al., 1985), was present at 19 nt upstream of the poly(A) tail (Fig. 3c).

Expression of WSSV-ts in E. coli

For the convenience of protein purification and identification, full-length WSSV-TS was expressed in E. coli as a fusion protein with an N-terminal His tag. The induced (plus IPTG at 37 °C) and non-induced samples were analysed by 14% SDS-PAGE (Fig. 4a). A band (about 32 kDa) corresponding to rTS protein was observed in the induced sample when compared to the sample without induction. The soluble rTS was purified by Ni–NTA affinity chromatography under native conditions, and was found to match the theoretical molecular mass of 32-6 kDa. Purified rTS was used for antibody preparation and the identification of function.

Transcription and expression analyses of WSSV-ts in vivo

RT-PCR was performed to detect ts-specific transcripts at different infection stages (0 to 72 h p.i.). The WSSV-ts transcript was first, slightly, detected at 4 h p.i. and maximally at 24 h p.i.; consequently, it was considered an early transcriptional gene of the WSSV genome. The WSSV-ts transcriptional pattern was similar to some other early WSSV genes (Van Hulten et al., 2000; Tsai et al., 2000; Chen et al., 2002). When RNA was treated with RNase and then subjected to RT-PCR with ts-specific primers, no RT-PCR amplicon was seen, indicating that no virus genomic DNA was left in the prepared RNA (data not shown).

To investigate expression of WSSV-ts in vivo, samples extracted at various times up to 72 h p.i. from hepatopancreas of infected crayfish were analysed by Western

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**Fig. 2.** Phylogenetic analysis of WSSV-TS performed using 27 TSs and 15 DHFR–TSs from different organisms including mammals, fungi, bacteria, protozoa and DNA viruses. The phylogenetic tree was drawn using DNAMAN software. WSSV-TS (indicated with *) grouped more closely to DHFR–TSs from protozoa than to those from other organisms.
Identification of WSSV-TS

WSSV is a major pathogen with a broad host range, high infectivity and causes high mortality. Approximately 181 open reading frames (ORFs) have been revealed by analysing the genomic DNA sequence and nearly 20% of them, including WSSV067, are found without a typical polyadenylation signal (AATAAA) downstream of the ORFs (Yang et al., 2001). Results from the transcriptional analysis showed that WSSV-ts mRNA was indeed polyadenylated, although the polyadenylation pathway is still not well defined. The consensus sequence (CGTGTTAG), which is required for efficient formation and processing of poly(A) tails in 67% of mammalian mRNA 3'9 termini (McLauchlan et al., 1985), is presumed to contribute to this unknown signal pathway. However, our findings provide the first example that WSSV genes without the typical polyadenylation signals could be polyadenylated using another poly(A) signal pathway during their transcription.

It has been reported that dUMP and folate are attached to the highly conserved TS binding sites to form a ternary complex during the catalytic reactions. The major amino acid residues for which a functional role has been reported are present within these binding sites. X-ray analysis of the TS crystal structure reveals that several key residues found in these motifs are essential for this reaction (Carreras & Santi, 1995). For instance, in E. coli TS, the residues N177 and Y181, which are located in a consensus sequence PFNIAS of the dUMP-binding site (motif 3), are involved in determining pyrimidine specificity and in dUMP-binding activity (Hardy & Nalivaika, 1992; Schiffer et al., 1995). In addition, the aspartate residue (D221) in human TS, in the consensus sequence DMGLGVP in motif 3, is involved in folate cofactor binding and catalysis (Chiericatti & Santi,

**DISCUSSION**

WSSV is a major pathogen with a broad host range, high infectivity and causes high mortality. Approximately 181 open reading frames (ORFs) have been revealed by analysing the genomic DNA sequence and nearly 20% of them, including WSSV067, are found without a typical polyadenylation signal (AATAAA) downstream of the ORFs (Yang et al., 2001). Results from the transcriptional analysis showed that WSSV-ts mRNA was indeed polyadenylated, although the polyadenylation pathway is still not well defined. The consensus sequence (CGTGTTAG), which is required for efficient formation and processing of poly(A) tails in 67% of mammalian mRNA 3'9 termini (McLauchlan et al., 1985), is presumed to contribute to this unknown signal pathway. However, our findings provide the first example that WSSV genes without the typical polyadenylation signals could be polyadenylated using another poly(A) signal pathway during their transcription.
By analysing the primary sequence of WSSV-TS, as shown in Fig. 1, the same consensus regions (i.e. containing N201, Y205 and D193 residues) were also found in WSSV-TS. These highly conserved motifs and residues imply that WSSV-TS has a similar or even the same structure as other TS, and consequently make it possible for it to perform the same biochemical functions.

It has been reported that dUMP binding to, and folate cofactors of, TS cause a major conformational change that converts the enzyme from the open form to a closed form of the ternary complex and results in spectroscopic changes (Carreras & Santi, 1995) with maximum absorbance at ~330 nm and ~265 nm and minimum absorbance at ~295 nm (Lockshin et al., 1984). UV difference spectroscopic analysis using rTS revealed a maximum absorbance at 322 nm and a minimum absorbance at 295 nm, which corresponded to the characteristics of the ternary complex. This presumably reflected changes of chromophores of folate and TS. This difference spectrum suggested that the WSSV-TS protein had the capacity to form a ternary complex in the presence of dUMP and folate.
The TS of WSSV plays a key role in the virus dTTP synthetic system by providing sufficient dTMP for dTTP production. Consequently it has a close relationship with viral DNA replication and proliferation by regulating the balanced supply of dTTP for normal DNA metabolism in collaboration with dUTPase (WSV112), ribonucleotide reductase (WSV172) and thymidylic kinase (WSV395), which are all encoded by the WSSV genome and expressed at the early stage of infection (Tsai et al., 2000; Van Hulten et al., 2000; our unpublished data). The identification of WSSV-TS thus provides us with a new research direction in the prevention and treatment of white spot syndrome disease.

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