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# White Spot Syndrome Virus (WSSV) Ribonucleotide Reductase (RR) is Differentiated from the Cellular RRs by its Insensitivity to Allosteric Inhibition by dTTP and dATP

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## ABSTRACT

The ribonucleotide reductase (RR) of white spot syndrome virus (WSSV) consists of two nonidentical subunits, proteins RR1 and RR2, which are both required for activity. Increased WSSV RR activity has been detected in WSSV-infected shrimp cells and in the baculovirus/insect expression system. In this report, the recombinant WSSV RR (rWSSV-RR) expressed in a baculovirus/insect cell system is characterized by coupling the reduction with a DNA polymerase reaction. We found that EDTA and hydroxyurea inhibited rWSSV-RR activity. This suggests that metal ions and free radicals are required for its activity, which is similar to the situation with eukaryotic cellular RRs. The reduction of cytidine 5'-diphosphate (CDP) catalyzed by the recombinant WSSV RR (rWSSV-RR) was accelerated by ATP, a general activator of RR. However, WSSV RR is differentiated from the cellular RRs by its insensitivity to allosteric inhibition by dTTP and dATP.

***Key words***: *WSSV, ribonucleotide reductase, allosteric regulation*

## INTRODUCTION

White spot syndrome (WSS) is a major viral disease that affects most of the commercially cultured and captured marine shrimp species and crabs globally (Chou *et al*., 1995; Lo *et al*., 1996a, b; Lotz, 1997). Its causative agent, white spot syndrome virus (WSSV); Nimaviridae; (see http://ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm for more information on this new family), is a double-stranded DNA virus that consists of an envelope and a rod-shaped nucleocapsid (Inouye *et al*., 1994; Nakano *et al*., 1994; Wang *et al*., 1995). Infection of shrimp cells with WSSV results in the production of several viral enzymes involved in DNA synthesis and metabolism (van Hulten *et al*., 2001; Tsai *et al*., 2000a, b; van Hulten and Vlak, 2001; Chen *et al*., 2002; Lin *et al*., 2002). These enzymes may play an important role in enabling viral infection of the cells even during the resting stage, and in increases the speed of DNA replication.

DNA synthesis is dependent on a supply of deoxyribonucleoside triphosphates (dNTPs). The only route for *de novo* synthesis of these precursors is provided by ribonucleotide reductase (RR), an enzyme that catalyzes the reduction of ribonucleoside diphosphates (NDPs) to their corresponding deoxyribonucleoside diphosphates (Reichard, 1993; Jordan and Reichard, 1998). RR exists in all eukaryotic and prokaryotic organisms. Several large DNA viruses-including varicella-zoster virus (Heineman and Cohen, 1994), herpes simplex virus (Cohen, 1972), pseudorabies

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virus (Lankinen *et al*., 1982), African swine fever virus (Cunha and Costa, 1992), baculovirus (van Strien *et al*., 1997) and vaccinia virus (Slabaugh *et al*., 1984)-encode their own RRs. Mammalian and some bacterial RRs as well as those produced by most DNA viruses exist as complexes of two nonidentical subunits. The holoenzyme consists of two copies of each subunit, both of which are necessary for activity (Jordan and Reichard, 1998).

Eukaryotic and some bacterial RRs are subject to allosteric regulation, best understood in bacteria (Reichard, 1993; Jordan and Reichard, 1998), which involves nucleotides as both positive and negative effectors. However, some viral RRs are not affected by the presence of intracellular nucleotides (Berglund, 1972; Langelier *et al*., 1978; Ponce *et al*., 1977; Slabaugh and Mathew, 1984). In this paper we characterize the activity of the WSSV recombinant RR activity produced by an insect baculovirus expression system and investigate its allosteric effectors by coupling the RR assay with a DNA polymerase reaction (Jong *et al*., 1998). In a previous study (Lin *et al*., 2002), we showed that WSSV RR had enzymatic activity. Here we further show that it can be distinguished from cellular RR by its insensitivity to allosteric inhibition by dTTP and dATP. This particular pattern of insensitivity has been reported for very few viruses, and is yet another demonstration of the uniqueness of the WSSV genome.

## Material and Methods

### Cells and Virus.

*Spodoptera frugiperda* (Sf9) cells were cultured in Sf900-II insect serum free medium (Gibco, BRL). *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV) was used as wild-type (wt) virus. Routine cell culture maintenance and virus infection were carried out according to published procedures with slight modification. The recombinant baculovirus AcMNPV-rr1 and AcMNPV-rr2 (Lin *et al*., 2002) was amplified by infection of Sf9 at a multiplicity of 0.5-1 PFU/cell at 27°C for 5-7 days, and the supernatant was collected and stored at 4°C as the virus stock.

### Recombinant protein expression.

The Sf9 insect cells ($3\times10^6$) were infected by AcMNPV-rr1 or AcMNPV-rr2 virus solution with 5-10 M.O.I. for 3 days at 27°C, and then the cells were harvested by centrifugation at 1,000 g for 15 min at 4°C. The supernatants were discarded, and the cell pellets were stored at -20°C until use.

The cell pellets from one 25 $cm^2$ T-flask were washed with buffer I [500 mM Hepes, pH 7.2; 2 mM dithiothreitol (DTT)], and then resuspended in a new aliquot (0.5 ml) of buffer I with RNase A. The suspension was sonicated (three 10-s bursts) and centrifuged at 20,000 g for 20 min. The supernatant was loaded onto a Sephadex G-25 column (~4 ml) equilibrated with buffer II (50 mM Hepes, pH 7.2; 2 mM DTT), and then 2 ml of buffer I was added to elute the column. Fractions of 0.5 ml were collected from each column. The concentration of total soluble proteins in each fraction was determined using Bio-Rad protein assay solution. The fraction containing soluble proteins was used for RR activity assay. For recombinant WSSV RR assay, the fractions of rRR1 and rRR2 were pooled.

### Enzyme activity assay.

The enzyme activity assay was performed as previously published (Jong *et al*., 1998) with slight modification. Unless otherwise specified, the standard RR assay solution (80 μl) contained 80-100 μg of desalted cell extract, 50 mM Hepes, pH 7.2, 6 mM DTT, 4 mM Mg acetate, 2 mM ATP, and 50 pmol [$^{14}C$] CDP. The reaction mixture was incubated at 37°C for 30 min and boiled for 5 min. It was then centrifuged for 5 min to remove the precipitates. The supernatant was

mixed with the labeling mixture, which contained 90 mM Hepes (pH 6.6), 10 mM $MgCl_2$, 0.2 mM dNTP, 150 ng/μl herring DNA, 1× random primer (Roche, Mannheim, Germany) and 5 units of Klenow fragment. Followed by incubation at room temperature for 30 min, the mixture was spotted onto a piece of 1.5-$cm^2$ Whatman DE-81 filter paper. The air-dried filter was washed twice with 5% $Na_2HPO_4$ for 5 min, once with methanol (5 ml/filter), and once with acetone (5 ml/filter). The washed filters were air-dried and inserted into vials containing 3 ml of a scintillation solution, FluoranSafe 2 Scintran (BDH Poole, Dorset, England). Counting of radioactivity was performed in a Packard Liquid Scintillation Analyzer Tri-Carb 1900TR.

## RESULTS

### Effects of EDTA and $Mg^{2+}$

Eukaryotic, some bacterial and viral RRs use dinuclear iron centers in the RR2 subunits to generate the catalytically essential tyrosyl radicals. Metal-chelating agents, such as EDTA, can destroy the diferric centers and thus make the enzyme inactive. In the presence of 10 mM, 20 mM and 40 mM of EDTA, the total RR activity of the AcMNPV-rr1 and AcMNPV-rr2 infected Sf9 cell lysate decreased almost to 50%, 20% and 10%; respectively (Fig. 1a). The inhibitory effects of EDTA demonstrated the metal involvement in the catalytic reaction.

Magnesium ion ($Mg^{2+}$) was required by cellular RRs for the association of two subunits, but some viral RR activities were inhibited by $Mg^{2+}$ or were unaffected by $Mg^{2+}$ (Cunha and Costa, 1992; Ponce *et al.*, 1977). In the presence of 2 mM ATP, the total RR activity of the AcMNPV-rr1 and AcMNPV-rr2 infected Sf9 cell lysate was optimized at 1 mM of $Mg^{2+}$, and then gradually dropped to 80% of the control (Fig. 1b). In the presence of 10 mM $Mg^{2+}$, the RR activity was still higher than 80%, which represents the RR activity in the

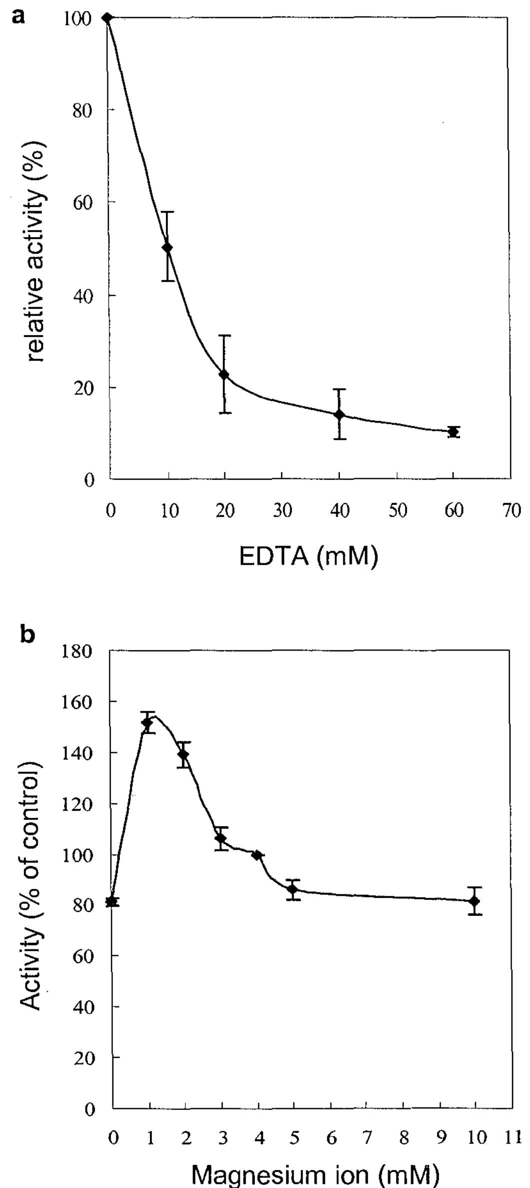


**Fig. 1.** Effects of EDTA and $Mg^{2+}$ on the total RR activity of the AcMNPV-rr1 and AcMNPV-rr2 infected Sf9 cell lysate. The relative RR activity (i.e., the percentage RR activity in the presence of various concentrations of effectors, [a] EDTA and [b] $Mg^{2+}$, with respect to that in the absence of EDTA or present of 4 mM $Mg^{2+}$) indicated on the ordinate is the combined (mean) results of three independent assays.

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absence of $Mg^{2+}$. These data indicate that $Mg^{2+}$ is not required for our RR assay system, but it is necessary for optimal RR activity.

### Allosteric regulation

The overall enzymatic activity and substrate specificity of *E. coli* and mammalian RRs are allosterically regulated (Jordan and Reichard, 1998). Often ATP is a positive effecter, and dNTPs are negative regulators of the overall RR activity. However, some virus-induced RRs showed different allosteric regulation from the cellular RRs. ATP was not required for HSV-2 and pseudorabies virus RR by date (Bapat *et al.*, 1987; Lankinen *et al.*, 1982) nor did it inhibit African swine fever virus RR (Cunha and Costa, 1992). In the presence of 4 mM $Mg^{2+}$, 2 mM of ATP enhanced the total RR activity of the AcMNPV-rr1 and AcMNPV-rr2 infected Sf9 cell lysate (Fig. 2a). In order to test the optimal concentration of ATP in the presence of 4 mM $Mg^{2+}$, the amounts of ATP in the RR assay solutions were varied. When 0.5 mM of ATP was present, the total RR activity increased to three times of that in the absence of ATP. The maximal activity occurred at 1 mM ATP; however, higher concentrations (2-10 mM) of ATP did not further promote but dropped back to the level in the absence of ATP (Fig. 2b).

The rWSSV-RR substrate specificity regulated by dATP was also examined. Addition of 1 mM dATP to the RR assay solutions severely depressed the RR activity from Sf9 cells to 35%, but accelerated the reduction of CDP catalyzed by the AcMNPV-rr1 and AcMNPV-rr2 infected Sf9 cell lysate to 132% that of the control (Fig. 3a). The data indicated WSSV RR and Sf9 RR were allosterically regulated by dATP in different ways.

In the presence of 1 mM dTTP, 53% of Sf9 RR activity was inhibited, but only 27% of RR activity was inhibited in the AcMNPV-rr1 and AcMNPV-rr2 infected Sf9 cell lysate (Fig. 3b). The Sf9 RR

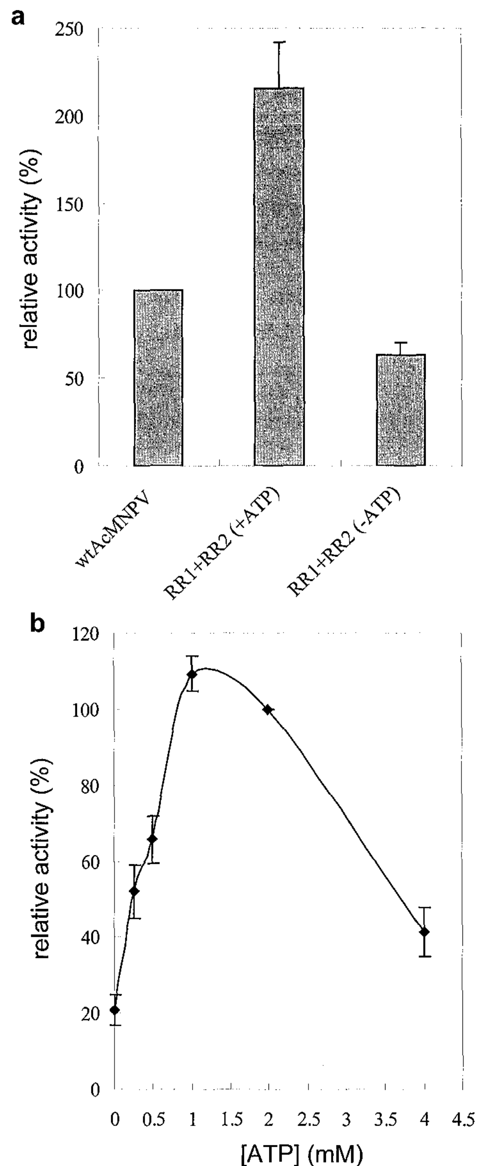


**Fig. 2.** Effect of ATP on the RR activity (a) in desalted cell extracts prepared from wtAcMNPV infected Sf9 cells and AcMNPV-rr1 and AcMNPV-rr2 infected Sf9 cells. Data show the relative RR activity (i.e., the percentage RR activity with respect to that in wtAcMNPV infected Sf9 cell control). (b) Various amounts of ATP were added to the RR assay solution. Data show the relative RR activity (i.e., the percentage RR activity with respect to that at 2 mM of ATP) and are the combined results of three independent assays.

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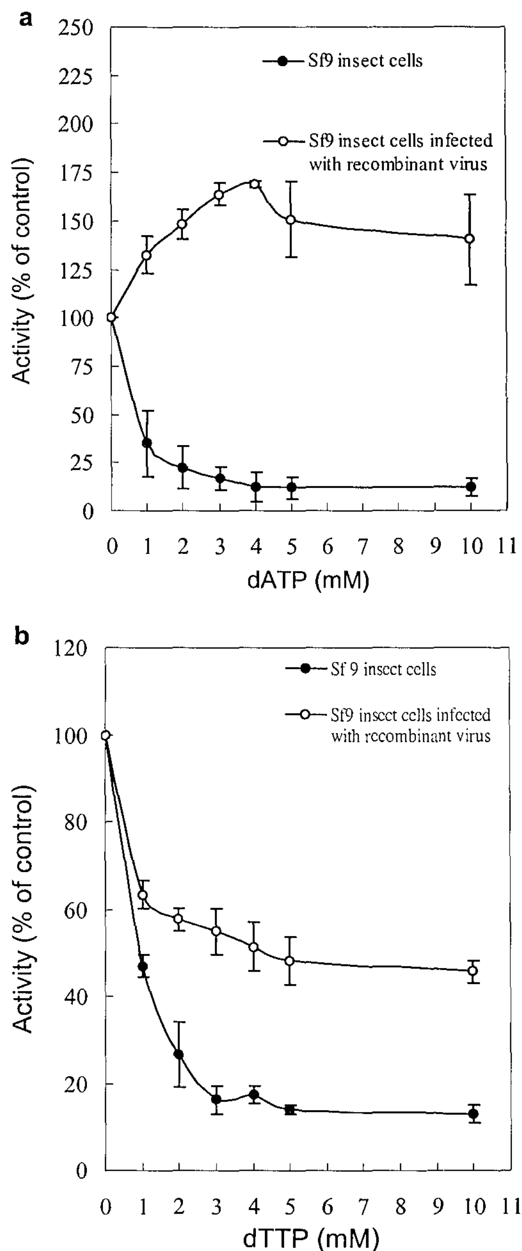


**Fig. 3.** Allosteric effects of dATP and dTTP on Sf9 RR and rWSSV-RR. Desalted cytoplamic extracts were assayed in the presence of various concentrations of (a) dATP and (b) dTTP.

activity was much more sensitive to the feedback inhibition by dTTP than that of WSSV RR.

## Inhibitory effect of hydroxyurea on WSSV RR

Hydroxyurea (HU) is known to be an RR2-specific inhibitor that quenches the free radical in the RR2 subunit of RR and inactivates its enzymatic activity (Thelander *et al.*, 1985). To test rWSSV-RR's sensitivity to HU, various concentrations of HU were added into the RR assay solutions. In the presence of 10 mM HU, the total RR activities of Sf9 cell lysate and the AcMNPV-rr1 and AcMNPV-rr2 infected Sf9 cell lysate decreased to 39% and 60% of the control, respectively (Fig. 4). Even in presence of 100 mM of hydroxyurea, the total RR activities of the AcMNPV-rr1 and AcMNPV-rr2 infected Sf9 cell lysate only diminished 55% of the control (data not shown). It suggested that WSSV RR is more resistant to HU than Sf9 RR.

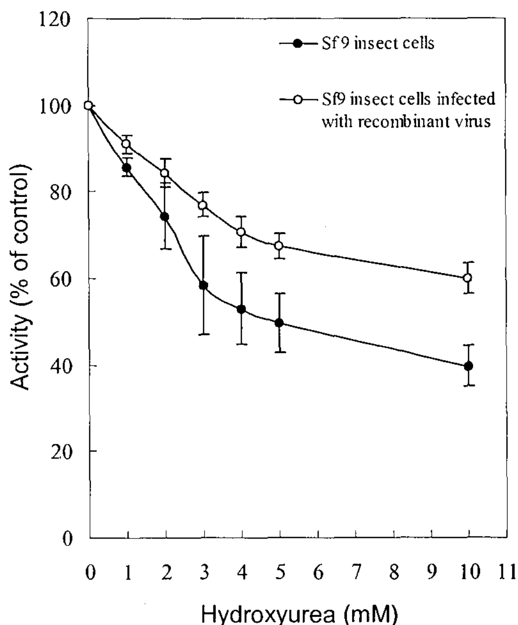


**Fig. 4.** Effect of hydroxyurea on Sf9 RR and rWSSV-RR expressed in a baculovirus/ insect cell system. Desalted cytoplamic extracts were assayed in the presence of various concentrations of hydroxyurea.

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## DISCUSSION

WSSV-infected shrimp cell exhibits a novel RR, in addition to the endogenous cellular enzyme (Lin *et al.*, 2002). *E. coli* and eukaryotic cellular RRs are tightly regulated by allosteric effectors, but some viral RRs are not regulated in the same way. The RR activities from shrimp cells and WSSV might be differentiated on the basis of their susceptibility to inhibition by dATP and dTTP, their requirement for $Mg^{2+}$, and their response to ATP. Since expression levels of WSSV RR in infected shrimp cells are too low to obtain sufficient purified protein for characterization, we therefore set up a baculovirus/insect cell system to characterize the total RR activities of Sf9 cell lysate and the AcMNPV-rr1 and AcMNPV-rr2 infected Sf9 cell lysate, and to examine their allosteric effects.

WSSV RR is similar to the cellular and herpes simplex virus type 1 (HSV-1) enzymes in using NDPs as substrates. It is also qualitatively similar in the inhibition by EDTA and HU, although there are quantitative differences between the enzymes. For example, perhaps because the diferric center is deeply buried inside the protein (Eklund *et al.*, 2001), 10 mM of EDTA was needed for inhibition of 50% RR in the AcMNPV-rr1 and AcMNPV-rr2 infected Sf9 cell lysate (Fig. 1a). This $I_{50}$ value is much higher than that of HSV-1 induced RR, 20 μM (Averett *et al.*, 1983).

WSSV RR is clear distinguished from Sf9 cellular RRs by its lack of negative regulation by dATP and dTTP (Fig. 3). It has already been showed that although uninfected Sf9 cells produce RR, infection by wtAcMNPV does not affect the levels of RR production (Lin *et al.*, 2002). This suggests that wtAcMNPV does not produce its own RR. At various concentrations of dTTP, the decreases (0-55%) of the relative RR activities shown by the AcMNPV-rr1 and AcMNPV-rr2 infected Sf9 cell lysate were actually contributed by Sf9 RR because the RR activities from cells infected by AcMNPV and from those not infected were almost equal (Lin *et al.*, 2002). In addition to WSSV RR, the RRs encoded by bacteriophage T4, Pseudorabies virus and by HSV-1 are also insensitive to dATP and dTTP inhibition (Berglund, 1972; Ponce *et al.*, 1977; Langelier *et al.*, 1978; Huszar and Bacchetti, 1981; Lankinen *et al.*, 1982; Averett *et al.*, 1983), but the RRs of vaccinia virus and ASFV are sensitive to dATP and dTTP inhibition (Slabaugh *et al.*, 1984; Cunha and Costa, 1992).

Although WSSV RR lacks negative regulation, it was still subjected to dATP positive regulation. Our previous data suggests that WSSV RR is a class I RR (Lin *et al.*, 2002). The RR1 subunit of *E. coli* class Ia RR contains two allosteric sites for binding effectors. One is the activity site, which regulates catalytic activity, and the other is the specificity site, which regulates substrate specificity. If ATP and dATP bind to the activity site and the specificity site, respectively, the reduction of CDP and UDP is enhanced (Reichard, 2002). Therefore, dATP, which enhances the reduction of pyrimidine nucleotides, such as UDP and CDP (Fig. 3a), becomes a purely positive allosteric effector for WSSV RR.

ATP acts as a general activator for most RRs, including that encoded by bacteriophage T4 (Hendricks and Mathews, 1997). ATP was required for optimization of WSSV RR activity, but not for HSV-1 RR activity (Averett *et al.*, 1983). It suggests that the ATP requirement is different in viral RRs. Many enzymes, like cellular RRs, at metabolic key points are feedback regulated by end products. In WSSV genome, the chimeric polypeptide of cellular-type thymidine kinase and thymidylate kinase was identified (Tsai *et al.*, 2000b) and characterized recently (Tzeng *et al.*, 2002). WSSV TK-TMK is inhibited by dTTP, which suggests that it is a cellular-type TK and TMK. In contrast, WSSV RR was not inhibited by dTTP. This result suggests that WSSV TK-TMK and RR are from two different origins.

RR is a critical enzyme for DNA

metabolism and DNA synthesis (Jordan and Reichard, 1998). RR is also tightly regulated in the cell cycle, closely coupled to cell DNA replication, and is absent or inactive in resting cells (Nordenskjöld *et al*., 1970). Ribonucleotide reductase is an important target for antiviral (Prichard and Shipman, 1995) and antiparasite (Ingram and Kinnaird, 1999) chemotherapy. However, WSSV attacks almost all cells within a short period. In this paper, in which we have shown that WSSV RR does need the dinuclear iron centers and the tyrosyl radicals for catalytic activity, and the insensitivity of negative regulation by dATP, dTTP may explain why WSSV replicates efficiently in old/resting cells as well as in young/actively proliferating cells.

## ACKNOWLEDGEMENTS

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# 蝦白點症病毒與真核細胞之核醣核苷酸還原酶對 dTTP 及 dATP 的異位調控具有不同敏感度

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蝦白點症病毒核醣核苷酸還原酶具有大、小兩個次單元，均為組成完整具有活性的酵素所需。今利用已經建立的核醣核苷酸還原酶活性分析系統，進而分析重組白點症病毒核醣核苷酸還原酶在功能上的需求，發現 EDTA 及核醣核苷酸還原酶小次單元抑制物 Hydroxyurea 都會對酵素有所抑制，因此推測與一般細胞中的核醣核苷酸還原酶相同，金屬離子與自由基是維持酵素活性的必要條件；此外，重組白點症病毒核醣核苷酸還原酶對 ATP 有所需求，但濃度過高或過低，都會有抑制的情形發生；在分析 dATP 及 dTTP 對於重組蝦白點症病毒核醣核苷酸還原酶活性的影響，得到的結果與昆蟲細胞的核醣核苷酸還原酶作比較，兩者對 dATP 及 dTTP 的反應並不相同，因此推測重組蝦白點症病毒核醣核苷酸還原酶與一般細胞的核醣核苷酸還原酶在回饋抑制的敏感度並不相同。

**關鍵詞**：蝦白點症病毒，核醣核苷酸還原酶，異位調控。

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