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Identification of white spot syndrome associated baculovirus (WSBV) target organs in the shrimp *Penaeus monodon* by *in situ* hybridization

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ABSTRACT: *Penaeus monodon* non-occluded baculovirus III (PmNOB III) is a strain of white spot syndrome associated baculovirus (WSBV) isolated from diseased black tiger shrimp *P. monodon*. In order to identify the target organs of WSBV, the tissue sections of experimentally infected shrimp sampled at 0, 16, 22, 40, 52, and 64 h post-infection were hybridized *in situ* with PmNOB III DNA-specific probe labeled with digoxigenin. WSBV-positive cells were initially observed at 16 h post-infection in the stomach, gill, cuticular epidermis and hepatopancreas. At 22 h post-infection, the lymphoid organ, antennal gland, muscle tissue, hematopoietic tissue, heart, midgut and hindgut were found to be WSBV-positive. The nervous tissue and compound eyes did not have WSBV-positive cells until 40 h post-infection. Thus various tissues from the mesoderm and ectoderm, such as connective tissue, epithelium, nervous tissue and muscle, can all be infected by WSBV. By 52 or 64 h post-infection, it was found that the stomach, gill, cuticular epidermis, lymphoid organ, hematopoietic tissue and antennal gland were all heavily infected with WSBV and that these tissues had become necrotic. The white spot syndrome itself first appeared at 40 h post-infection, and the shrimp began to die at 64 h post-infection. The probe demonstrated no reaction to WSBV-negative tissue.

KEY WORDS: PmNOB III · WSBV · *In situ* hybridization · White spot baculovirus · Penaeid shrimp

INTRODUCTION

White spot syndrome associated non-occluded baculovirus (WSBV) is the causative agent of a shrimp viral disease. The affected individuals always show white spots on their exoskeleton and this is the principal sign of the disease (Chou et al. 1995, Wang et al. 1995). Based on the published description, WSBV is also apparently related to Japan's rod-shaped nuclear virus of *Penaeus japonicus* (RV-PJ; Inouye et al. 1994, Momoyama et al. 1994, Nakano et al. 1994, Takahashi et al. 1994), China's hypodermal hematopoietic necrosis baculovirus (HHNBV; Cai et al. 1995) and Thailand's systemic ectodermal and mesodermal baculovirus (SEMBV; Wongteerasupaya et al. 1995).

WSBV is extremely virulent, has a wide host range and targets various tissues. The known hosts of WSBV include *Penaeus japonicus*, *P. monodon*, *P. chinensis* and *P. penicillatus* (Cai et al. 1995, Wang et al. 1995). According to a study by Momoyama et al. (1994) on the histopathology of RV-PJ-infected *P. japonicus*, degenerated cells with basophilic hypertrophied nuclei were observed in various tissues originating from both mesoderm and the ectoderm, such as the cuticular epidermis, connective tissue, lymphoid organ, antennal gland, hematopoietic tissue and nervous tissue (Momoyama et al. 1994). The virus can induce 100% mortality in infected shrimp within 3 to 5 d (Nakano et al. 1994). Since 1993 the virus has been a significant pathogen in cultured shrimp in Asia.

The virions of *Penaeus monodon* non-occluded baculovirus III (PmNOB III), which is a strain of WSBV isolated from diseased black tiger shrimp *P. monodon*,

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have been purified. In negatively stained preparations, the virion measures 70 to 150 nm at its broadest point and is 250 to 380 nm long, which is usually 10% larger than in ultrathin sections (Wang et al. 1995). A PmNOB III genomic DNA library was also constructed and a set of specific primers was designed for the polymerase chain reaction (PCR) to detect PmNOB III infection in penaeid shrimp (Lo et al. 1996). However, the sites of early viral infection and the stages of the progression of WSBV infection through the shrimp remained unknown.

This paper reports on the use of a PmNOB III DNA-specific probe to identify by *in situ* hybridization the target organs and tissues of WSBV in experimentally infected shrimp. With this technique, we could identify the major tissues which were sites of initial viral infection as well as the preferred attack sites of WSBV. We were also able to follow the stages of the progression of WSBV infection in shrimp. The results of our current studies will provide important insights into the pathogenic mechanism, early diagnosis and disease control.

MATERIALS AND METHODS

Experimental infection. Samples of *Penaeus monodon* were obtained from a shrimp farm at Kaohsiung in Taiwan. They were originally determined to be not infected by PmNOB III by PCR detection. Detection of the virus by PCR followed the methods of Lo et al. (1996). Three groups of about 30 shrimps weighing 0.35 to 0.45 g were kept in 50 l plastic tanks containing salinity 20 ppt, filtered, aerated sea water at ambient temperature (approximately 28 to 30°C). Two of these groups were fed with severely PmNOB III-infected *P. monodon* for one meal and then with artificial diet for the subsequent days. The third group of shrimp served as a negative control and were fed with artificial diet throughout the study.

Sampling procedure and treatment. Three shrimp from each group were sampled at 0, 16, 22, 40, 52 and 64 h post-infection (pi). These shrimp were fixed in buffered neutral formalin solution (10% formalin, 33 mM NaH_2PO_4, 45 mM Na_2HPO_4). The fixed tissues were dehydrated, embedded in paraffin wax and sectioned at approximately 4 to 5 µm thickness on a rotary microtome. The sections were stained with modified hematoxylin and eosin (H&E stain) for normal histology and the sections from the same tissue blocks were also placed onto 2% silane pretreated slides for further *in situ* hybridization (Nuovo et al. 1991).

DNA probe for *in situ* hybridization. The recombinant plasmid pms146 selected from the PmNOBIII *Sal* I genomic library (Lo et al. 1996) was used for the preparation of the DNA probe. The insert of pms146 was gel purified and nonradioactively labeled with digoxigenin-dUTP using a random priming method available from Boehringer Mannheim Biochemical, Bedford, England.

***In situ* hybridization procedure.** The tissue sections were deparaffinized in xylene and rehydrated by a series of graded alcohols (absolute to 50%) and finally with distilled water The sections were soaked briefly in phosphate-buffered saline (PBS; 0.137 M NaCl, 0.0027 M KCl, 0.0043 M $Na_2HPO_4 \cdot 2H_2O$, 0.0014 M KH_2PO_4, pH 7.4) and then placed for 10 min in 0.02 N HCl. After washing twice for 5 min in PBS at room temperature (RT), the slides were treated for 30 min with 100 µg ml^{-1} proteinase K in PBS at 37°C. Following the proteolytic treatment, slides were washed twice for 5 min in PBS containing 0.2% glycine. Post-fixation was carried out by 4% paraformaldehyde in PBS for 10 min at RT, and then the sections were washed in 0.2% glycine in PBS for 10 min followed by a wash in 2× standard saline citrate (SSC; 1× SSC = 0.15 M NaCl, 0.015 M tri-sodium citrate, pH 7.0). These sections were pre-hybridized in 500 µl hybridization solution (50% formamide, 0.2% Ficoll 400, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin (BSA), 5× SSC, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA) for 1.5 h at 37°C. Digoxigenin-labeled DNA probes were diluted to 10 ng ml^{-1} in hybridization solution and 100 µl was applied to each slide. A cover glass was put on top and the slides were placed on a 95°C hot plate for 5 min. The slides were then transferred to a humid chamber and incubated at 37°C for 16 to 20 h. After hybridization, the slides were uncovered and washed by 2× SSC for 30 min at RT, followed by 1× SSC for 30 min at RT, 0.5× SSC for 30 min at 37°C and then 0.5× SSC for 30 min at RT.

Hybridization detection. The slides were washed briefly in Buffer I (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) and then blocked with Buffer I containing 1% blocking reagent (Boerhringer) (Buffer II) for 30 min at 37°C. The alkaline phosphatase-conjugated anti-digoxigenin was diluted 1:2500 in Buffer II. The slides were covered with 300 µl diluted conjugate and incubated for 30 min at 37°C in a dark, humid chamber After washing twice for 15 min with Buffer I containing 0.3% Tween-20, the slides were equilibrated for 5 min in Buffer III (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) at RT The slides were covered with 500 µl of freshly prepared color developer [45 µl nitro-blue tetrazolium (90 mM) and 35 µl 5-bromo-4-chloro-3-indoyl phosphate (120 mM) in 10 ml Buffer III] and were then incubated for 1 h or overnight at RT After incubation, the slides were washed with 1× TE buffer for 5 min at RT. Neutral red was used to counterstain the slides for 10 min and then they were dehydrated with a series of graded alcohols (80% to absolute),

before being finally immersed in xylene. Each slide was then mounted with a plastic lid by using a histological mounting medium and examined under an Olympus BX-50 bright field light microscope.

RESULTS

Infectivity and mortality of the experimental infection

The percentages of sampled shrimp with positive *in situ* hybridization are shown in Table 1. At 16 h pi, 33% of the shrimp showed a blue to purple precipitate in their tissues. At 22 h pi, 66% of the shrimp produced a positive signal and by 40 h pi all the sampled shrimps were found to be WSBV-positive by the *in situ* hybridization method. These percentages were similar to those of shrimp found positive by PCR. The white spot syndrome itself was first seen in the shrimp sampled at 40 h pi. The size and number of white spots increased subsequently with sampling time. No experimental shrimp died until 64 h pi, at which time the mortality of the remaining shrimp was 33%. Shrimp in the control group did not show any positive signal in their tissues by *in situ* hybridization or by PCR, nor did they have any white spots on their carapaces. All the shrimp in the control group were healthy and survived during the experimental period (data not shown).

Table 1. Detection of white spot syndrome associated non-occluded baculovirus in tissues of experimentally infected *Penaeus monodon* by *in situ* hybridization. pi: post-infection

	0 h pi	16 h pi	22 h pi	40 h pi	52 h pi	64 h pi
In situ hybridization[a]	0%	33%	66%	100%	100%	100%
White spot syndrome[b]	–	–	–	+	++	+++
Mortality[c]	0/30	0/27	0/24	0/21	0/18	5/15

[a]Percentage of positive shrimps in each sampled detected by *in situ* hybridization
[b]Relative amount and size of white spots on the carapace: (–) no white spot detected, (+) few and small, (++) many and small, (+++) many and large
[c]Mortality of remaining shrimps at each sampling

Table 2. Chronological appearance and staining intensity of white spot syndrome associated non-occluded baculovirus by *in situ* hybridization in tissues of experimentally infected *Penaeus monodon*. pi: post-infection. Relative intensity of positive staining and integrity of cell: (+) light blue, intact cell with normal nuclei; (++) bright blue, intact cell with hypertrophied nuclei; (+++) blue to purple, cell with disintegrated nuclear membrane; (++++) bright purple, cell lysed; (–) no colored precipitate detected

Tissue	0 h pi	16 h pi	22 h pi	40 h pi	52 h pi	64 h pi
Stomach	–	+	++	+++	++++	++++
Gill	–	+	++	++	+++	++++
Cuticular epidermis	–	+	+++	+++	++++	++++
Hepatopancreas	–	+	+	+	++	++
Heart	–	–	+	++	++	+++
Lymphoid organ	–	–	+	++	+++	++++
Hematopoietic tissue	–	–	+	++	+++	++++
Antennal gland	–	–	+	++	+++	++++
Mid- and hindgut	–	–	+	+	++	+++
Compound eye	–	–	–	+	++	++
Nervous system	–	–	–	+	++	++

Detection of WSBV by *in situ* hybridization

The degree of WSBV infection in the tissues was rated by the number of cells with blue-purple precipitate, the intensity of coloration and the integrity of cells on a scale of + to ++++. For those tissues that were intact and stained a light blue to purple in the nuclei, the rating of + was used. This corresponds to an early stage of infection. Intact tissues that were stained a bright blue and contained hypertrophied nuclei were given ratings of ++. Tissues in which the nuclear membrane had disintegrated and blue to purple coloration extended to the cytoplasm were rated +++. The tissues that were stained bright purple and showed obvious cytopathological changes were given a rating of ++++. Intact tissues in which no pigmentation was detected were given a rating of –.

The sequential progression of the virus to the organs is shown in Table 2. The light blue to purple precipitate was present in the nuclei of the columnar cuticular epithelial cells and spongy connective tissue of the stomach demonstrated at 16 h pi (Fig. 1A). These tissues were still intact and most of the cells did not show any cytopathological changes. The percentage of positive cells was not more than 10% at this time. At 22 h pi, the number of positive cells increased, the coloration turned deeper and hypertrophied cell nuclei began to appear (Fig. 1B). The degree of infection became more severe at 40 h pi (Fig. 1C). Even in the sections stained with H&E, it was easy to observe the hypertrophied nuclei (Fig. 1E). Some cell nuclei were enlarged to more than twice the diameter of a normal nucleus. After 52 h pi, the epithelium layer and connective tissue of stomach were strongly positive and many cells were lysed (Fig. 1D).

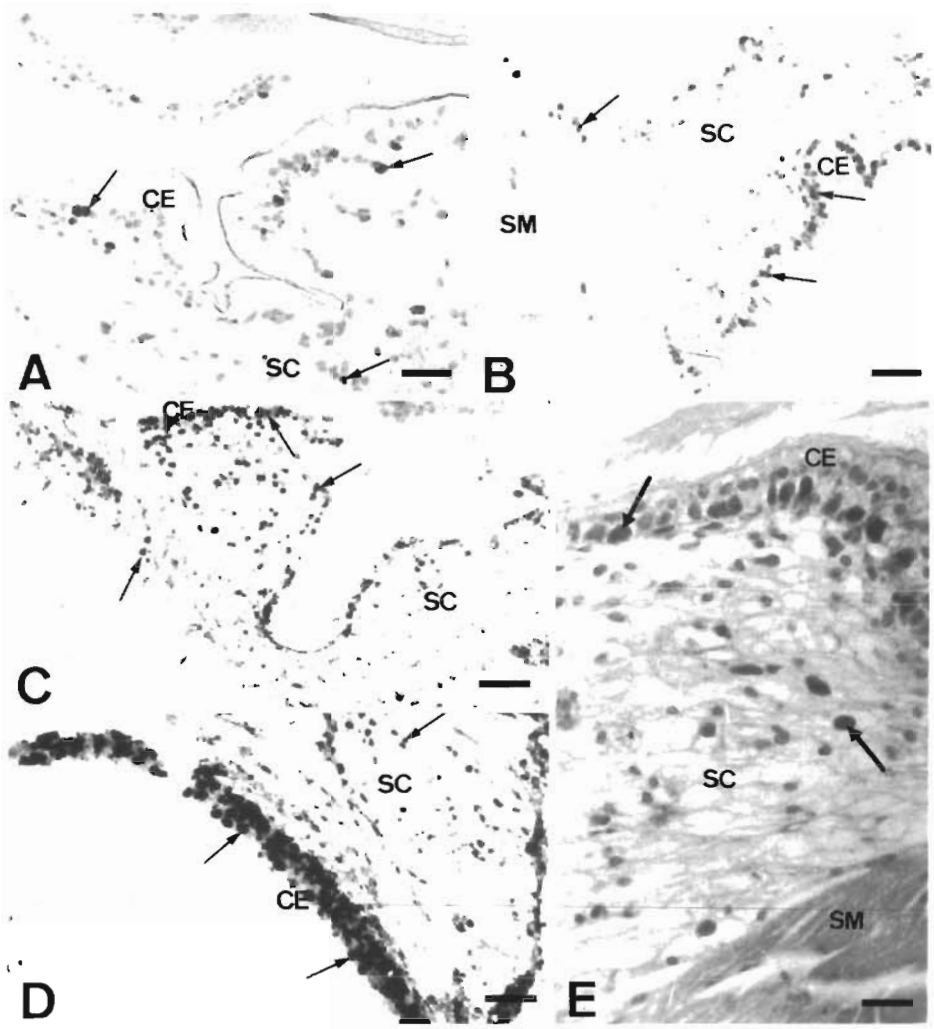

Fig. 1 Detection by *in situ* hybridization of white spot syndrome associated non-occluded baculovirus (WSBV) in the stomach of experimentally infected shrimp sampled at different times: (A) 16 h post-infection (pi), (B) 22 h pi, (C) 40 h pi, (D) 52 h pi. Positive cells (arrows) were located in the cuticular epithelium (CE) and spongy connective tissue (SC) but not in the smooth muscle layer (SM). Scale bar = 100 µm. (E) Severely affected epithelium in the stomach (H&E stain; 40 h pi). The nuclei of the CE cells and the SC are hypertrophied (arrows). Scale bar = 50 µm

In the gill, viral DNA was first seen at 16 h pi in the epithelium of the primary and secondary filaments. The number of positive cells was small and the degree of infection was low initially (Fig. 2A). The color of the precipitate became deep blue and cytopathological changes began to occur at 52 h pi (Fig 2B). At 64 h pi, more than half of the epithelial cells were virus-positive, contained hypertrophied nuclei and had become degenerate. The organ structure was also disintegrated as the area of the lacunaes were increased (Fig. 2C). Cells with hypertrophied nuclei were observed in the sections stained with H&E at this time (Fig. 2D).

The cuticular epidermis (including the epithelium of the eye stalk, appendages and the epithelium under the carapace) were also first observed to have purple precipitate at 16 h pi. These epithelial cells became severely degenerative and lysed after 52 h pi (Fig. 3).

In the hepatopancreas, the connective tissues and myoepithelial cells of the hepatopancreas sheath and in the intertubular spaces were positive at 16 h pi but the epithelium of the tubules was not (Fig. 4A). Cytopathological changes in the hepatopancreas occurred at 40 h pi. After 52 h pi, most of the cells in the intertubular region were strongly positive but the tubular epithelium was still almost completely negative (Fig. 4B, C).

The virus was first seen in the heart, muscle tissue, lymphoid organ, hematopoietic tissue, antennal gland, midgut and hindgut at 22 h pi. In the heart, positive cells were located in muscle fibers and connective tissue. After 64 h pi, about 40% of the muscle fibers and connective tissue yielded a deep blue precipitate and contained hypertrophied nuclei (Fig. 5A) In the muscle tissue, purple precipitate was present in the nuclei of the muscle fibers. At 64 h pi, the nuclei became hypertrophied and stained deep purple but the muscle fibers were still intact (Fig. 5B). In the lymphoid organ, many stromal matrix cells surrounding the lumen and the fibrous connective tissue were positive (Fig. 6A). These cells became lysed and the sheaths had degenerated by 64 h pi. The stem cells of the hematopoietic tissues were virus-positive (Fig. 6B).

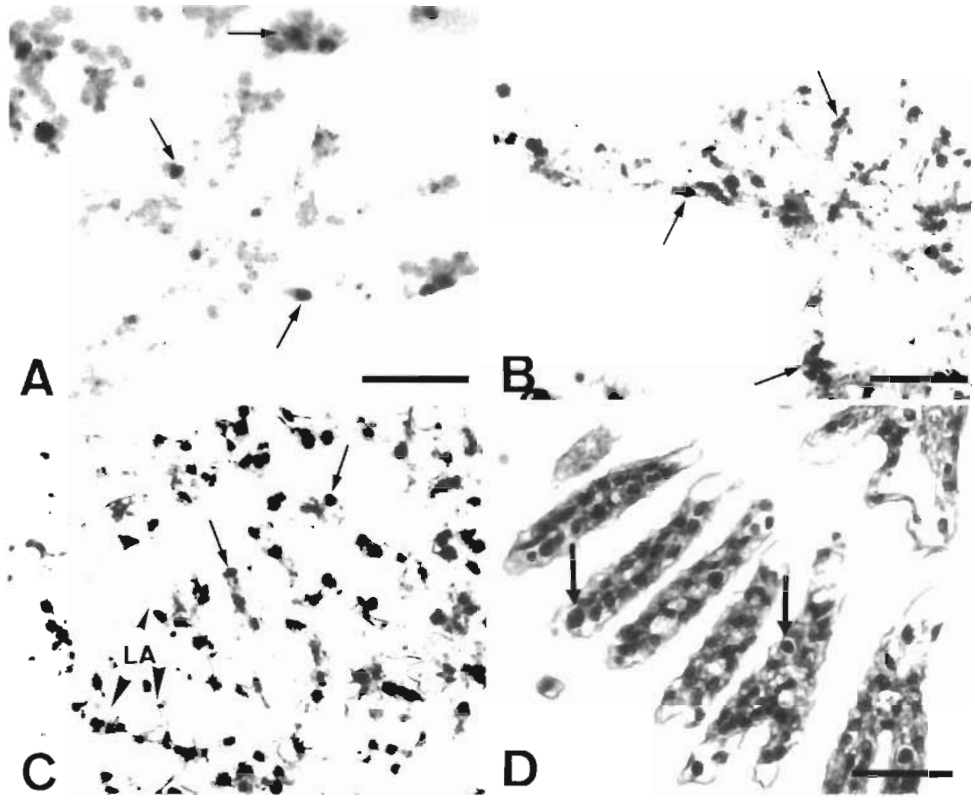

Fig. 2. Detection by *in situ* hybridization of WSBV in the gill of experimentally infected shrimp sampled at different times: (A) 16 h pi, (B) 52 h pi, (C) 64 h pi. Positive cells (arrows) were located in the epithelium of primary and secondary filaments. LA: lacunae (arrow head). Scale bar = 100 µm. (D) Severely affected epithelium in the gill (H&E stain; 64 h pi). The nuclei of the epithelial cells (arrows) are hypertrophied. Scale bar = 50 µm

In the late infection stage, hematopoietic tissue was severely necrotic because most cells were lysed. In the antennal gland, the positive cells were located in the epithelium surrounding the gland tubules. The structure of the tubules disintegrated during the late infection stage (Fig. 6C). Unlike the stomach, the positive cells were located in the muscle and underlying connective tissue region but not in the epithelium of the midgut and hindgut. However, it is hard to determine whether the positive signal was in the muscle cells or in the connective tissue or both. At 64 h pi, the nuclei of positive cells became hypertrophied and stained deep purple (Fig. 6D).

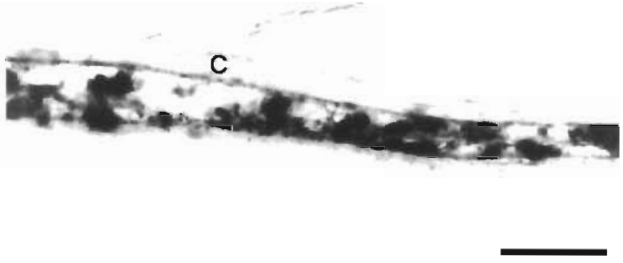

Fig. 3. Detection by *in situ* hybridization of WSBV in the pleopod of experimentally infected shrimp sampled at 52 h pi. Most of the epithelial cells were strongly positive and had hypertrophied nuclei. C: cuticle. Scale bar = 50 µm

The fasciculated zone and lamina ganglionaris of the compound eyes and the glial cells of the ganglia were initially viral DNA positive at 40 h pi. After 64 h pi, these tissues still showed only medium levels of infection (Fig. 7A, B).

DISCUSSION

The results demonstrated that a 1461 bp DNA fragment from the genomic library of PmNOB III can be used as a specific probe to detect WSBV in paraffin-embedded, fixed tissues of infected shrimp by *in situ* hybridization. The absence of any precipitate in tissues not infected by WSBV suggests that this probe does not cross-react with shrimp tissue. Recently, *in situ* hybridization techniques have been developed for some shrimp virus diagnosis, such as infectious hypodermal and hematopoietic necrosis virus (IHHNV; Mari et al. 1993), *Penaeus monodon*-type baculovirus (MBV; Poulos et al. 1994), *Baculovirus penaei* (BP; Bruce et al. 1993) and hepatopancreatic parvo-like virus (HPV; Mari et al. 1995). The occluded baculovirus can be detected in the tissues of infected shrimp before the occlusion bodies occur by *in situ* hybridization (Bruce et al. 1994). It is more advantageous to use *in situ* hybridization to detect viral DNA in shrimp tissue than to use histological staining, electron microscopy or

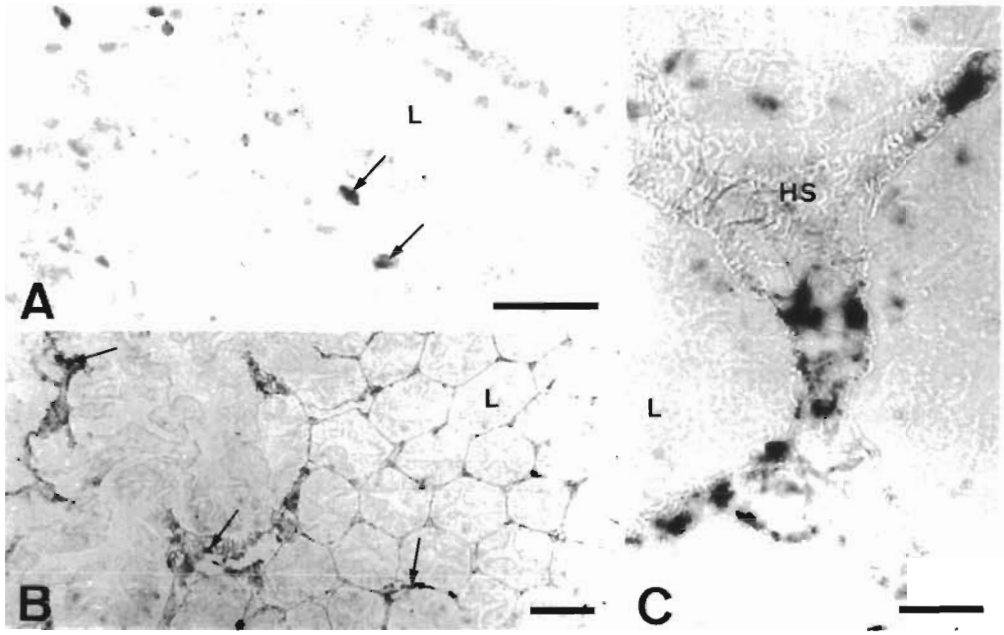

Fig. 4. Detection by *in situ* hybridization of WSBV in the hepatopancreas of experimentally infected shrimp sampled at (A) 16 h pi, (B & C) 64 h pi. Positive cells (arrows) were located in the surrounding connective tissue and intertubular connective and myoepithelial cells of the hepatopancreas. (C) High magnification photomicrograph to show the intertubular space of the hepatopancreas. L: lumen; HS: hemal sinus. Scale bar in (A) = 100 µm, (B) = 300 µm, (C) = 40 µm

PCR. Although the electron microscopy technique can definitely provide the information about the sites of viral distribution and the state of viral maturation at the cellular level, much time is required to obtain the results and expensive equipment is also required. *In situ* hybridization can accurately provide the precise location of viral DNA present in tissue sections as a result of the highly specific interaction between the probe and the target sequence of viral DNA. This property makes *in situ* hybridization superior to PCR, which cannot provide information about the target cell type within a given organ or tissue. Although WSBV does not form occlusion bodies, it can be easily detected by histological staining in late stages of infection. However, this may be more difficult in the lightly infected stage when cytopathological phenomena are still not obvious. *In situ* hybridization can overcome this problem.

The results of this study suggest that the cuticular epithelium from every part of the body of the shrimp is one of the main target tissues for the WSBV. The connective tissues of some organs are also infected by WSBV In other tissues such as nervous tissue, muscle tissue, lymphoid tissue and hematopoietic tissue, WSBV DNA can also be observed. In terms of the organs, WSBV does severe damage to the stomach, gills, hematopoietic tissue, lymphoid organ, antennal gland and cuticular epidermis of the shrimp. During the late stages of infection, these organs were destroyed and many cells were lysed. The organs which were more lightly infected by WSBV included the hepatopancreas, nerve node, compound eye, the muscle and connective tissue of midgut and hindgut. The degree of infection of these organs did not increase and they maintained organ integrity up to the late infection stage, although a few cells had cyto-

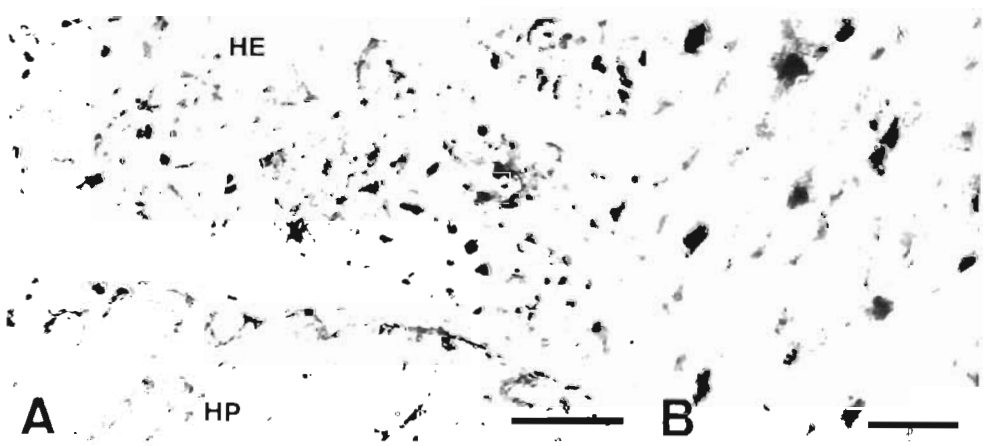

Fig. 5. Detection by *in situ* hybridization of WSBV in (A) the heart and (B) the muscle tissue of experimentally infected shrimp at 64 h pi Nuclei of muscle cells show a positive signal. HE: heart; HP: hepatopancreas. Scale bar in (A) = 200 µm, (B) = 50 µm

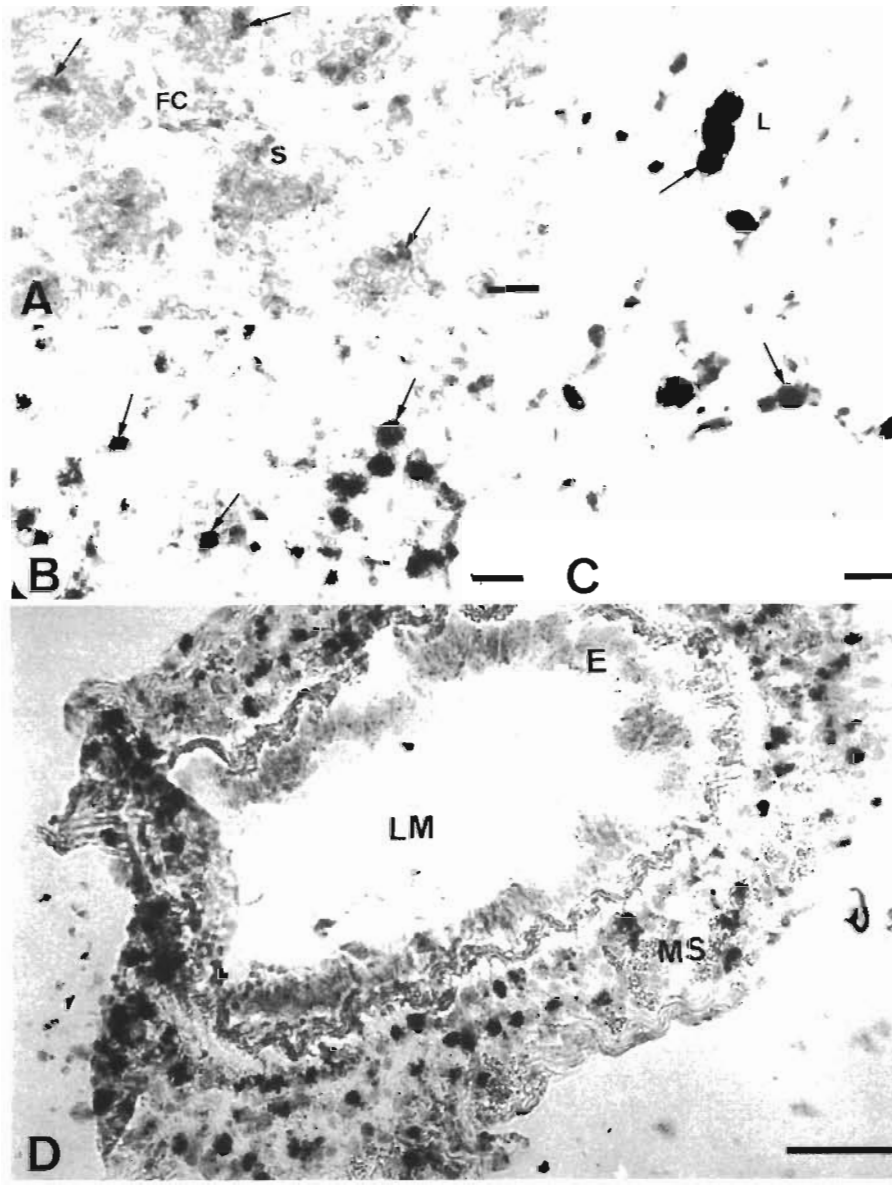

Fig. 6. Detection by *in situ* hybridization of WSBV in (A) the lymphoid organ, (B) the hematopoietic tissue, (C) the antennal gland, and (D) midgut of experimentally infected shrimp sampled at (A, B, C) 52 h pi and at (D) 64 h pi. Positive cells indicated by arrows. FC: fibrous connective tissue; E: epithelium; L: antennel gland lumen; LM: midgut lumen; MS: muscle; S: stromal matrix cell. Scale bar in (A) & (B) = 50 µm, in (C) = 25 µm, in (D) = 100 µm

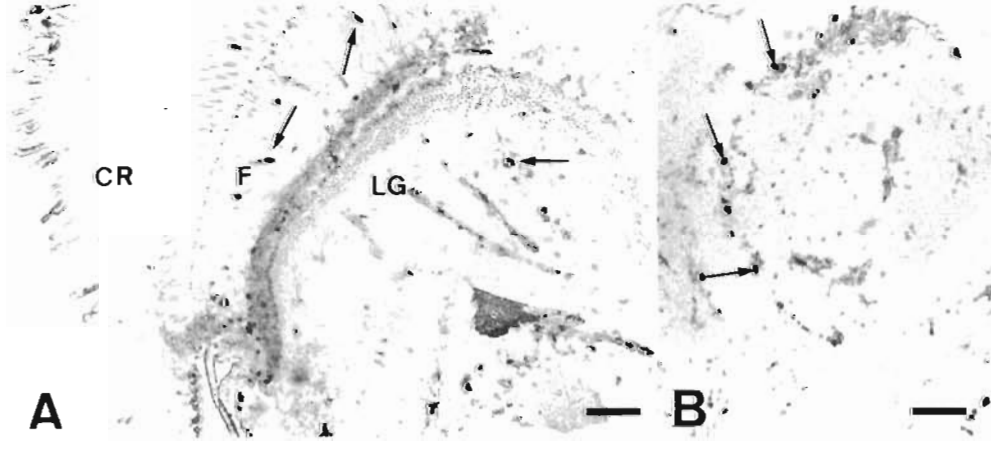

Fig. 7. Detection by *in situ* hybridization of WSBV in nervous tissues of experimentally infected shrimp sampled at 64 h pi. (A) compound eye. The positive cells (arrows) were located in the fasciculated zone (F) and lamina ganglionaris (LG). CR: crystalline tract. Scale bar = 100 µm. (B) Ganglion. The glial cells of ganglion were positive (arrows). Scale bar = 100 µm

pathological signs and were lysed. Thus, it can be concluded that WSBV can infect shrimp tissues that originate from the ectoderm or the mesoderm. This distinguishes WSBV from other shrimp baculoviruses such as MBV, BP and baculoviral midgut gland necrosis virus (BMNV) which only infect the epithelial cells of the hepatopancreas tubules and the midgut in shrimp (Momoyama 1983, Chang et al. 1992, 1993, Bruce et al. 1993, 1994).

According to the results of this study, although WSBV can also infect the hepatopancreas, it is mainly confined to the myoepithelial cells of the hepatopancreatic sheath and the fibroblasts of the connective tissue; there was little evidence, if any, that it infected the epithelium of the tubules. Some parts of the hepatopancreas were lysed in the late infection stage but no viruses were detected. This lysis was evidently not due directly to virus infection, but was probably due to the generally unhealthy state of the infected shrimp and resulted from autolysis. As for the enteric tissue, the time at which midgut and hindgut were first detected to be infected with WSBV was later than for the stomach and the degree of infection was also lighter than the degree of infection in the stomach.

The results also show that the virus initially appears in the shrimp in the stomach, gill, cuticular epidermis and connective tissue of the hepatopancreas. Chou et al. (1995) indicated that under experimental conditions the virus can infect the shrimp via water and oral inoculation. From this study, some shrimp sampled at 16 h showed virus in the stomach but not in the gills, while others sampled at the same time showed virus in the gills but not in the stomach (data not shown). This suggested that the virus infection could be either via the oral pathway or via water to the gill or cuticular epidermis. Since virus-infected shrimp tissues were given to the experimental shrimp as feed, both oral and waterborne infections were possible. In some sections, a few positive signals could be detected in the lumen of the stomach at the immediate onset of infection. Specifically, these signals were in the cuticle layer above the epithelium. This finding can be explained if the virus-containing feed had just entered the stomach lumen and some of it had adhered to the setal grooves of the cuticle layer. It may also result from a non-specific binding to chitin that has been noted by Lightner (1996).

WSBV can also infect the heart, eyes and nervous tissue of shrimp. The most severely infected site of the eyes was the cuticular epidermis of the eye stalk. Some nerve cells of the compound eyes and glial cells of the ganglia were also lightly infected by WSBV in the late stage. Changes found in WSBV-infected shrimps can be distinguished easily from the white eye syndrome in Taiwan in 1991 in terms of brown fibrous nodules in the fasciculated zone of the compound eyes (Chen et al. 1991).

In conclusion, a sensitive, specific and nonradioactive nucleic acid probe can be used for the *in situ* detection of the WSBV genome. Viral infection was detected at an early stage, the degree of infection was determined, and target tissues were identified. The route of WSBV entry and progression of the disease caused by WSBV was also established.

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