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A yellow head virus gene probe: nucleotide sequence and application for *in situ* hybridization

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ABSTRACT: A portion of the genome of yellow head virus (YHV) of penaeid shrimp was cloned and the cDNA fragment (1161 bp) was designated clone 3-27. The fragment was labeled with digoxigenin and hybridized *in situ* to tissue sections of YHV-infected *Penaeus vannamei*. Positively reacting tissues included those of the lymphoid organ, cuticular epithelium, and gills. In addition, connective tissue of hepatopancreas, heart, antennal gland, hematopoietic organ, nerve tract, midgut cecum and muscle reacted to the probe. The probe was highly specific since it hybridized only to tissues from YHV-infected shrimp. It did not react to those of uninfected shrimp or shrimp infected with WSSV (white spot syndrome virus), IHHNV (infectious hypodermal and hematopoietic necrosis virus), or TSV (Taura syndrome virus). The clone was sequenced, and primers were synthesized for rapid detection of YHV in hemolymph using RT-PCR (reverse transcription-polymerase chain reaction). The strand that constituted the viral sequence in the cDNA was also determined via RT-PCR and *in situ* hybridization with a single-stranded RNA (ssRNA) probe.

KEY WORDS: Penaeid shrimp · Yellow head virus · *In situ* hybridization · RT-PCR

INTRODUCTION

Yellow head virus was first observed in Thailand in the early 1990s, where it resulted in significant mortality and adversely affected the shrimp culture industry (Flegel et al. 1995). Other YHV-like viruses, such as gill associated virus (GAV) and lymphoid organ virus (LOV), have been reported from penaeid shrimp cultured in Australia (Spann et al. 1995, 1997). The proposed relationship between the Australian YHV-like viruses and YHV was based on histological changes in the infected shrimp and characteristics of the virions by electron microscopy.

YHV was named from the gross sign of a yellow cephalothorax in infected *Penaeus monodon*. However, other shrimp with YHV infection do not always exhibit a yellow cephalothorax. For example, YHV-infected *P. vannamei* and *P. stylirostris* do not display this sign, but they are pallid when moribund. Histopathological studies have shown that shrimp with YHV infection display a generalized multifocal to diffuse necrosis, with obvious nuclear pyknosis and karyorrhexis. Basophilic, spherical, cytoplasmic inclusions occur in affected tissues, especially in hemocytes, lymphoid organs, cuticular epithelium, gills, and connective tissue of several organs, including muscles, antennal glands, gonads, hematopoietic organs, nerve tracts, etc. (Lightner 1996).

Examination of thin sections of YHV-infected tissues by transmission electron microscopy (TEM) revealed the presence of enveloped, bacilliform virus particles measuring 160–186 × 38–50 nm in the cytoplasm of infected cells (Chantanachokin et al. 1993). TEM of negatively stained, purified YHV virions revealed spike-studded, enveloped particles measuring 150–170 × 40–50 nm (Wongteerasupaya et al. 1995). The complete virion has an electron-dense nuclear core that measures 20 to 30 nm in diameter and is surrounded by a trilaminar envelope (Nadala et al. 1997).

The YHV genome is RNase sensitive, which suggests that it is a single-stranded RNA (ssRNA) virus

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(Wongteerasupaya et al. 1995). Using SDS-PAGE gel to analyze viral structural proteins, 4 polypeptides of 170, 135, 67 and 22 kDa were found. The 135 kDa polypeptide was glycosylated and was likely to be the viral glycoprotein (Nalada et al. 1997). Based on size, bacilliform morphology, cytoplasmic replication, and the fact that it was an ssRNA virus, YHV was proposed to be a member of the *Rhabdoviridae* (Nadala et al. 1997).

Traditional methods for the diagnosis of yellow head disease are based on clinical signs and histopathology. These methods are not highly specific and are not useful for detecting the early stages of infection or asymptomatic carriers. Therefore, more sensitive and specific methods are needed. This report describes cloning of YHV genomic cDNA and its application as a gene probe for *in situ* hybridization. In addition, sequence information from the cDNA was used to develop an RT-PCR detection technique that employed aliquots of shrimp hemolymph as the source of viral RNA template.

MATERIALS AND METHODS

YHV isolate and propagation in penaeid shrimp. The isolate of YHV used in this study was obtained in 1993 from farm-raised *Penaeus monodon* in Thailand and maintained at the University of Arizona by continuous transfer in *P. vannamei*. The inoculum was prepared from a homogenate of infected tissues (cephalothoraces) in TN buffer (0.02 M Tris-HCl, pH 7.2, 0.4 M NaCl). The tissue homogenate was then diluted with 2% saline and injected into the muscle of healthy *P. vannamei*. The inoculated shrimp were maintained in seawater at 26 to 28°C. When the shrimp became moribund, hemolymph samples were drawn and frozen at −70°C for subsequent virus purification or RT-PCR. The cephalothoraces were fixed in Davidson's AFA (Bell & Lightner 1988) for 24 to 48 h then changed to 70% ethanol for storage prior to histological preparation.

Virus purification. Collected hemolymph was frozen and thawed 3 to 4 times to release cell-associated virions from hemocytes, then centrifuged to remove cellular debris at 3000 × *g* for 30 min at 4°C. The supernatant was centrifuged at 100 000 × *g* at 4°C for 1 h to pellet the virions. The viral pellet was resuspended in TN buffer and further purified on a 15 to 35% Renografin density gradient at 153 200 × *g* for 1 h. The viral band was visualized by top illumination and collected through a fraction collector. The fractions containing viral particles were centrifuged at 100 000 × *g* for 1 h to remove the Renografin. The virus pellet was resuspended in TN buffer. Purified viral particles were negatively stained on carbon-collodium-coated grids with sodium phosphotungstate (2%) and examined under a Philips CM 12S transmission electron microscope.

Viral RNA extraction and cDNA synthesis. Purified viral particles were mixed with a TRIzol LS reagent (Life Technologies, MD, USA) in accordance with the manufacturer's protocol. The purified RNA was resuspended in DEPC (diethylpyrocarbonate)-treated water and stored at −70°C. The amount of RNA was quantified by a spectrophotometer at UV_{260}. cDNA was synthesized with the SuperScript Choice System (Life Technologies). Prior to cDNA synthesis, the RNA was denatured at 70°C for 10 min, followed by a quick chilling on ice. Random hexamer was used to prime cDNA synthesis by SuperScript II RT (RNase H^- Moloney murine leukemia virus reverse transcriptase). The synthesis of the first strand of cDNA was performed at 42°C for 1 h. Then, the second strand of cDNA was synthesized with a mixture of *Escherichia coli* DNA polymerase I, RNase H and *E. coli* DNA ligase at 16°C for 2 h. *Eco*R I adaptors were added to the ends of the cDNA followed by ligation into the dephosphorylated *Eco*R I site of a plasmid (pSport I, Life Technologies). The recombinant plasmid was transformed into competent *E. coli* DH5α and clones containing cDNA inserts were selected with IPTG (isopropylthio-β-D-galactoside) and Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) in accordance with standard procedures (Sambrock et al.1989).

DNA sequencing. Purified plasmid DNA was sent to the DNA Sequencing Lab, Division of Biotechnology, University of Arizona. DNA was sequenced with a PRISM Ready Reaction DiDeoxy Terminator Cycle Sequencing kit from Applied Biosciences.

DNA labeling. The inserted cDNA fragment was labeled with digoxigenin-11-dUTP in a PCR reaction. The labeling mixture included 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.165 mM dTTP, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.035 mM digoxigenin-11-dUTP, 2 mM $MgCl_2$, 0.3 µM primer, template DNA (1 ng $µl^{-1}$), and 50 U ml^{-1} of AmpliTaq Gold (Perkin-Elmer, NJ, USA). The primers were YHV1051F (5'-ACA TCT GTC CAG AAG GCG TC-3') and YHV-1051R (5'-GGG GGT GTA GAG GGA GAG AG-3'). The amplification was performed in a thermocycler (GeneAmp 480, Perkin-Elmer) with the following cycle parameters: initial denaturation at 94°C for 10 min, followed by 50 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and a final extension at 72°C for 7 min. The digoxigenin-labeled DNA probe was precipitated with ethanol, resuspended in H_2O, and stored at −20°C.

***In situ* hybridization.** Cephalothoraces of YHV-infected shrimp were fixed in Davidson's AFA for 24 to 48 h. Alternatively, shrimp were fixed in a neutral-buffered, modified Davidson's fixative (called RF-fixative) (Hanson et al. 1997). The fixed shrimp were processed and embedded in paraffin using standard histological methods. Four µm thick sections were cut,

and mounted on Superfrost Plus slides (Fisher Scientific, PA, USA). Prior to hybridization, the sections were incubated at 65°C for 45 min, then deparaffined in Hemo de (Fisher Scientific), and rehydrated through an ethanol series to water. Sections were digested with proteinase K (100 µg ml^{-1}, in 50 mM Tris-HCl, pH 7.4, 10 mM NaCl 1 mM EDTA) for 15 min at 37°C, followed by postfixation in formaldehyde (0.4%) for 5 min. The sections were rinsed in 2× SSC (standard saline citrate), then prehybridized with 500 µl of hybridization solution (4× SSC, 50% formamide, 1× Denhardt's, 0.25 mg ml^{-1} yeast RNA, 0.5 mg ml^{-1} salmon sperm DNA, 5% dextran sulfate) at 42°C for 30 min. For hybridization, the sections were overlaid with 250 µl of hybridization solution containing a digoxigenin-labeled probe (20 to 40 ng ml^{-1}) at 42°C overnight. The next day, sections were washed as follows: 2× SSC once for 30 min at room temperature; 1× SSC twice for 5 min at 37°C; 0.5× SSC twice for 5 min at 37°C. The sections were then incubated with sheep anti-digoxigenin-alkaline phosphatase conjugate (Boehringer Mannheim, Germany) at 37°C for 30 min. They were then washed with 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl twice for 10 min at room temperature and rinsed with 0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl. The sections were incubated with NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro-3-indoyl phosphate) in the dark for 1 to 2 h. After color had developed, the sections were counter-stained with Bismarck Brown Y (0.5%), dehydrated through a series of ethanol and Hemo de, and then mounted with coverslips and Permount (Fisher Scientific). Tissue sections from WSSV-infected shrimp were heat-treated for 10 min at 80°C after the addition of probe.

Reverse transcription and PCR (RT-PCR). The one-tube GeneAmp EzrTth RNA PCR kit (Perkin-Elmer) was used for RT-PCR. Total RNA extracted from hemolymph was incubated at 70°C for 10 min and reverse transcribed at 60°C for 30 min. The PCR reaction was initiated at 94°C for 2 min, followed by 40 cycles of PCR (94°C for 45 s, and 60°C for 45 s) ending with 60°C for 7 min. Primers used for the RT-PCR were oligonucleotides YHV273F (5'-CAA GAT CTC ACG GCA ACT CA-3') and YHV273R (5'-CCG ACG AGA GTG TTA GGA GG-3'). These primers generated a predicted 273 bp DNA fragment after PCR. An aliquot of the RT-PCR product was analyzed by 1.6% agarose gel electrophoresis.

For determination of the viral sequence, hemolymph was collected from YHV-infected shrimp and centrifuged at 4000 × *g*) for 15 min to remove hemocytes and cell debris. RNA was extracted from the cell-free supernatant with TRIzol LS reagent, resuspended in the DEPC-treated water and stored at −70°C. One primer (either YHV273F or YHV273R) was used during the RT reaction at 72°C for 30 min, followed by 94°C inactivation of the reverse transcriptase activity for 2 min. The other primer was then added and 40 cycles of PCR were then performed as described above.

Preparation of digoxigenin-labeled ssRNA probes and *in situ* hybridization. Digoxigenin-labeled RNA probe was synthesized with digoxigenin-11-UTP (Boehringer Mannheim) and a MAXIscript kit (Ambion, TX, USA) in accordance with the supplied protocol. The *Pst* I-linearized plasmid DNA from clone 3-27 was used with SP6 RNA polymerase to synthesize one strand, and the *Sma* I-linearized plasmid was used with T7 RNA polymerase to create the opposite strand. *In situ* hybridization with the digoxigenin-labeled ssRNA probes was performed at 50°C for overnight with the same hybridization buffer described for *in situ* hybridization using the digoxigenin-labeled double-stranded DNA probe. The concentration of digoxigenin-labeled ssRNA probe was 40 ng ml^{-1}. The posthybridization washes were carried out as follows: 2× SSC once for 30 min at room temperature; RNase A (20 µg ml^{-1}, in 2× SSC) digestion for 30 min at 37°C; 1× SSC twice for 5 min at 37°C; 0.5× SSC twice for 5 min at 37°C. The subsequent reaction with sheep anti-digoxigenin-alkaline phosphatase conjugate and color detection were the same as described above.

RESULTS

YHV purification and cloning of YHV genomic cDNA

YHV isolated from hemolymph of infected shrimp was density-gradient purified and negatively stained for TEM examination. The virions were shown to be rod-shaped and enveloped with spike projections on the surface. They appeared to be very fragile since many were damaged and frequently fragmented into 2 pieces. The size of the intact virions measured 154 to 210 nm by 50 to 57 nm. The mean size was approximately 179 × 54 nm. These measurements were not rigorously calibrated and this may account for them being slightly larger than those of Wongteerasupaya et al. (1995).

We isolated the YHV genomic RNA from purified viral particles using a single-step guanidium isothiocyanate-acid phenol procedure (i.e. TRIzol LS reagent). The cDNA was synthesized and cloned into the plasmid, pSport I. After cDNA cloning, over 300 clones were collected. A pair of pUC/M13 primers in a PCR reaction was used to determine the size of the cloned cDNA inserts. One clone contained an insert of 1.1 kb and was designated 3-27. It was labeled with digoxigenin-11-dUTP in a PCR reaction using pUC/M13 primers, so the labeled DNA fragment included an

additional 234 bp from the cloning vector (pSport I). This labeled cDNA fragment was used as a probe for *in situ* detection of YHV. It hybridized to YHV-infected shrimp tissues and not to tissues of uninfected shrimp. This clone was subsequently sequenced since it appeared to contain YHV genomic cDNA.

Sequence of clone 3-27 cDNA insert

The insert of clone 3-27 was characterized by restriction enzyme mapping and nucleotide sequencing (Fig. 1). The sequence of the 1161 bp insert was compared with that of a YHV sequence previously published (135 bp, Wongteerasupaya et al. 1997) and it was found that 84 bp at the end (close to the T7 promoter site) of the 3-27 cDNA insert overlapped with the reported 135 bp cDNA fragment.

In situ hybridization with a digoxigenin-labeled dsDNA YHV probe

A pair of primers (YHV1051F/R) was designed from the sequence of the YHV cDNA insert to prepare a 1051 bp labeled YHV cDNA probe by PCR. This digoxigenin-labeled probe did not contain any sequence from the cloning vector. It was hybridized *in situ* to tissue sections of YHV-infected shrimp. In general, the lymphoid organ, cuticular epithelium and gills showed the most pronounced positive hybridization reactions. Intense blue-purple precipitates appeared in the outer periphery of stromal matrix cells in the lymphoid organ tubules, but none appeared in the inner layer of stromal matrix cells or in the lumen-lining, endothelial cells. The connective tissue between the tubules also exhibited a positive reaction to the YHV probe (Fig. 2A). H&E staining of lymphoid organs revealed multiple foci of necrosis marked by pyknotic and karyorrhectic nuclei (Fig. 2B). In the stomach, the cuticular epithelium and underlying spongy connective tissue reacted to the YHV probe. The epithelial cells appeared to give a stronger positive reaction, in terms of intensity, than did the subcuticular connective tissue cells (Fig. 2C). The reaction of muscle cells surrounding the stomach was negative, but connective tissue in the muscle bundles reacted positively (data not shown). In gill tissues, afferent/efferent vessels and epithelial and pillar cells of gill filaments gave strong positive reactions (Fig. 2D).

The connective tissues associated with several organs examined were positive. In the hepatopancreas (Fig. 2E), the tubules did not react with the probe, but the myoepithelial cells of interstitial connective tissues that surrounded the tubules were positive and the connective tissue capsule of the hepatopancreas was also positive. In the heart (Fig. 2F), the myocardial cells were negative, but the connective tissue surrounding the heart was positive. Fixed phagocytes were also positive to the probe. In the antennal gland (Fig. 2G), epithelial cells in the tubules gave a negative reaction, while surrounding fibrous connective tissues were positive. In the nerve tracts (Fig. 2H), glial cells reacted to the YHV probe. In the hematopoietic organ (Fig. 2I), connective tissues were positive, while the parenchymal cells were negative. Also, epithelial folds of the midgut cecum (Fig. 2J) were negative and only submucosal connective tissue was positive. No positive reaction was seen in any of the tissues prepared from healthy, uninfected shrimp or from the TSV-, WSSV-, or IHHNV-infected shrimp.

RT-PCR

Another pair of primers (designated as YHV273F/R) was designed from the YHV cDNA insert to generate a 273 bp DNA fragment after RT-PCR. For this RT-PCR, a total RNA was isolated from hemolymph of YHV-infected shrimp and it generated a predicted 273 bp DNA fragment (Fig. 3). Hemolymph without prior RNA purification was also used successfully as a template to generate the 273 bp DNA fragment in RT-PCR reactions. The 273 bp PCR fragment was not obtained with hemolymph from uninfected shrimp.

Determination of viral strand sequence

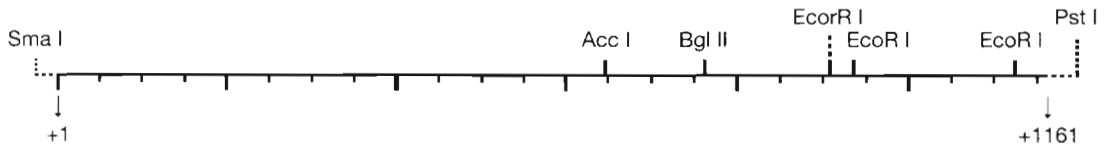
The primers YHV273F/R were used in RT-PCR reactions to determine which strand of the cDNA constituted a copy of the viral strand. When RNA isolated from cell-free hemolymph was used as the viral RNA template, only primer YHV273R annealed to the viral RNA and initiated the synthesis of cDNA for the subsequent successful PCR reaction to generate the characteristic 273 bp YHV amplicon. No amplicon was obtained when primer YHV273F was used in the RT step of the reaction (Fig. 4). The T_m values of primers YHV273F and YHV273R were very close (64.1°C and 63.7°C, respectively) so that differential annealing of YHV273R and YHV273F to the RNA template could not be used to explain the difference in the RT-PCR result for the 2 primers. In addition, positive *in situ* hybridization (Fig. 5A) occurred only with a digoxigenin-labeled ssRNA probe product from T7 RNA polymerase (same sequence as the bottom strand of YHV cDNA shown in the Fig. 1B but with U substituted for T). There was negative *in situ* hybridization (Fig. 5B) with an ssRNA probe product synthesized by SP6 RNA polymerase (i.e. the strand with a sequence

the same as the upper strand of cDNA shown in the Fig. 1B but with U substituted for T). Both the RT-PCR results and the *in situ* hybridization results indicated that the upper strand of cDNA shown in Fig. 1B corresponded to the viral sequence.

Sequence analysis

The 3-27 YHV genomic cDNA sequence was translated into 6 reading frames. The viral strand (i.e. the upper strand shown in the Fig. 1B) contained relatively

A



B

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   1  TCAAGATGGA CATCTTCGAC TACACCTACA CCTGCTACAC AAAAGCACAG TCATTCGCAT
      AGTTCTACCT GTAGAAGCTG ATGTGGATGT GGACGATGTG TTTTCGTGTC AGTAAGCGTA

  61  TACAAGCATG TACAATATAC AACTTCGACA TCACTCCAGA CAACATCTGT CCAGAAGGCG
      ATGTTCGTAC ATGTTATATG TTGAAGCTGT AGTGAGGTCT GTTGTAGACA GGTCTTCCGC

 121  TCTATGACTT CGAGACATAT CGTCCCGGCA ATTGTGATCC CATCAAAGCT CTCAACGCCG
      AGATACTGAA GCTCTGTATA GCAGGGCCGT TAACACTAGG GTAGTTTCGA GAGTTGCGGC

 181  TCACGTATTG CATCGAACGT CACTGGTTCT CCGCTGGTCT CTCACTCTCC TGCGCATCTA
      AGTGCATAAC GTAGCTTGCA GTGACCAAGA GGCGACCAGA GAGTGAGAGG ACGCGTAGAT

 241  TTTACCCACA TGAAGACATG ACAATTCATC AGTACAAAGA AGCATTCGCA CTCTACACTA
      AAATGGGTGT ACTTCTGTAC TGTTAAGTAG TCATGTTTCT TCGTAAGCGT GAGATGTGAT

 301  CAGAATTGAA CACAGAAGTC ACTCTCAAAC ACCAACCTAC TTTCGACTCC TATCTCAACT
      GTCTTAACTT GTGTCTTCAG TGAGAGTTTG TGGTTGGATG AAAGCTGAGG ATAGAGTTGA

 361  TCATGCTTGT CACAGAGCGT CACAACATCA ACATTGACAT CGGCACAGGG AGCAGACACC
      AGTACGAACA GTGTCTCGCA GTGTTGTAGT TGTAACTGTA GCCGTGTCCC TCGTCTGTGG

 421  TTCTACACTT CCTTCGACAA CATCACATCA GCTCCGTGTA CAGAAGAACG ATACAACGAA
      AAGATGTGAA GGAAGCTGTT GTAGTGTAGT CGAGGCACAT GTCTTCTTGC TATGTTGCTT

 481  GTCATGGCCG GGGTTACTCG CTTATATTAT GCCTACCAGT ATGATAGAGG TGATTTTCCT
      CAGTACCGGC CCCAATGAGC GAATATAATA CGGATGGTCA TACTATCTCC ACTAAAAGGA

 541  TGCAAATACA CAGTCACTCA AACTCACGTC AAATACCCTG TAATTGGCGA TGTTGCTGTG
      ACGTTTATGT GTCAGTGAGT TTGAGTGCAG TTTATGGGAC ATTAACCGCT ACAACGACAC

 601  GAACCTGAAG AATGCAAAAA TCCGATCTGC AACAGTTACC CACCAGTCTA CAGTGCTCTG
      CTTGGACTTC TTACGTTTTT AGGCTAGACG TTGTCAATGG GTGGTCAGAT GTCACGAGAC

 661  ATCTCCATCC AGAAATTCAG CACCTGGGCT CGTCTCATGT GTCATGATAT TCTCAAGCGA
      TAGAGGTAGG TCTTTAAGTC GTGGACCCGA GCAGAGTACA CAGTACTATA AGAGTTCGCT

 721  GTCTTCAATC ACTGTCGTGA CTGTGACCAT CTTAACTGCA AGATCTCACG GCAACTCATG
      CAGAAGTTAG TGACAGCACT GACACTGGTA GAATTGACGT TCTAGAGTGC CGTTGAGTAC

 781  CGCTTCAAGA ATCCACTTTC CAACATTCAG CCAGTCGCAT ACACTAAACT ACACGAAGAT
      GCGAAGTTCT TAGGTGAAAG GTTGTAAGTC GGTCAGCGTA TGTGATTTGA TGTGCTTCTA

 841  CGTCACCTAA TCCGTGACCG TCTCACACAC ATGGACTTCA CCTCAGGTCA AGAGTTCTAC
      GCAGTGGATT AGGCACTGGC AGAGTGTGTG TACCTGAAGT GGAGTCCAGT TCTCAAGATG

 901  GCTACTGAAT TCATCAATGA GTTCAATGAC GTCGAATTCA AGACACTGAA CGGTGATACC
      CGATGACTTA AGTAGTTACT CAAGTTACTG CAGCTTAAGT TCTGTGACTT GCCACTATGG

 961  ATTCACTTCA ACCTCACGCA TCCCTATGAC GCTCTCCTCG AACCTCTACT TCCTCCTAAC
      TAAGTGAAGT TGGAGTGCGT AGGGATACTG CGAGAGGAGC TTGGAGATGA AGGAGGATTG

1021  ACTCTCGTCG GAAATTCAGT GGCAGCATCA GGCGTTATTA GTGATCTTGA CGCTAATTTC
      TGAGAGCAGC CTTTAAGTCA CCGTCGTAGT CCGCAATAAT CACTAGAACT GCGATTAAAG

1081  AAAAACTACG ACAGAAACAC CGGCATGTCC TGTTCTCTCA CTGAATTCCA GCTCTCTCCC
      TTTTTGATGC TGTCTTTGTG GCCGTACAGG ACAAGAGAGT GACTTAAGGT CGAGAGAGGG

1141  TCTACACCCC CTACTCTTTC C
      AGATGTGGGG GATGAGAAAG G
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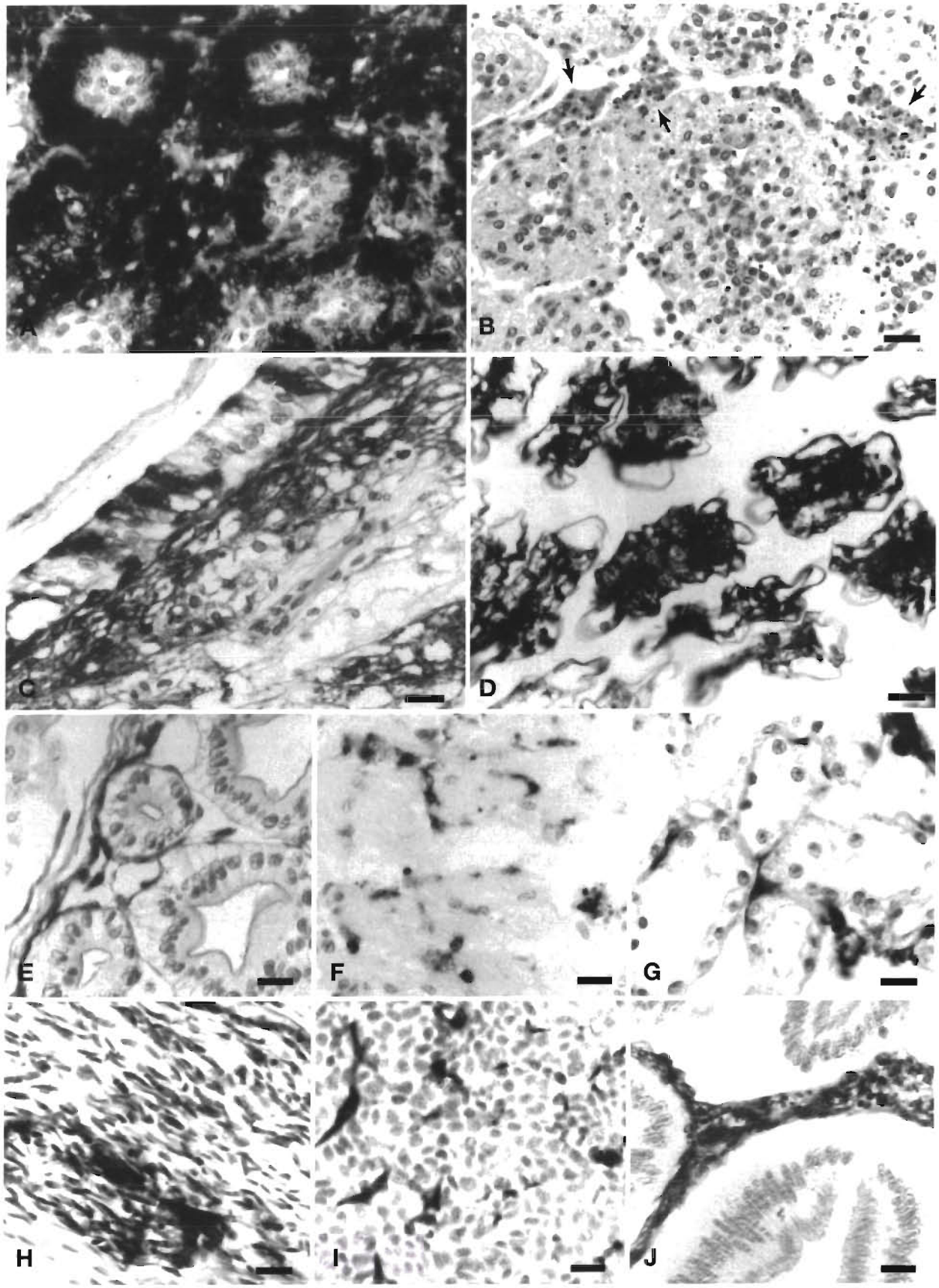
Fig. 1. (A) Restriction enzyme map and (B) nucleotide sequence of the cDNA insert in clone 3-27. (A) The YHV cDNA insert was digested with *Acc* I, *Bgl* II and *Eco*R I. The positions of restriction sites are *Acc* I at +647, *Bgl* II at +761, and *Eco*R I at +907, +934, and +1123. Sites of *Sma* I (–25) and *Pst* I (+1192) were located upstream and downstream of the cDNA insert, respectively. (B) The SP6 (–119) and T7 (+1230) promoters reside upstream and downstream, respectively, of the starting site (+1) of the cDNA insert. Nucleotides in bold type (**N**) represent sequences of the PCR primers YHV1051F/R. Underlined nucleotides (N) are the primers YHV273 F/R


A
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H
I
J

Fig. 2. *In situ* hybridization of tissue from YHV-infected shrimp with a digoxigenin-labeled dsDNA probe. (A) lymphoid organ; (B) H&E stained lymphoid organ, with several foci (arrows) containing pyknotic and karyorrhectic nuclei seen throughout the tissue; (C) cuticular epithelium of the stomach; (D) gill tissue; (E) hepatopancreatic tissue; (F) heart; (G) antennal gland; (H) nerve tract; (I) hematopoietic organ; (J) midgut cecum. The DNA probe was labeled with digoxigenin by PCR using primers YHV1051F/R with the cDNA insert in clone 3-27 as template. Scale bars = 20 µm

large open reading frames (226 amino acids [aa] for frame 1 from nucleotide 484 to 1161 and 172 aa for frame 3 from nucleotide 6 to 524). The strand complementary to the viral strand contained no open reading frame larger than 100 aa. This suggested that the viral strand was a sense strand, if the sequenced portion of the YHV genome encoded for a polypeptide. Using the Blast program of Seqlab software (GCG, Genetics Computer Group) to search for homologous sequences in DNA and protein sequence databases, no homologous viral or invertebrate sequences were found.

DISCUSSION

This report describes the cloning of a portion of YHV genomic cDNA and its successful application as a highly specific gene probe to detect YHV infection by *in situ* hybridization. The *in situ* hybridization results were consistent with those based on traditional histology but were more sensitive in that cells with no visible signs of infection by normal histology could be detected with the probe. The use of the digoxigenin-labeled dsDNA probe coupled with alkaline phosphatase detection was simple and practical and the non-radioactive probe could be stored for long periods. The colored precipitate in positively reacting tissues lasts several months without significant fading and the low background allows for unambiguous determination of reaction sites.

In situ hybridization clearly revealed YHV tissue tropism. The lymphoid organ, gills, and cuticular epithelial cells gave the highest viral signal, while connective tissues gave moderate signals. Although the mechanism of YHV tissue tropism is not known, our results

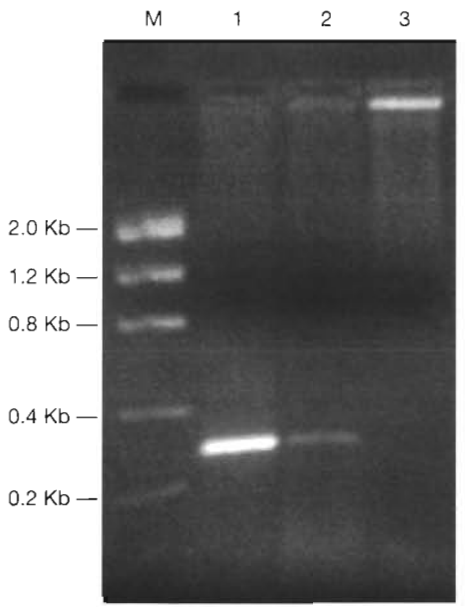

Fig. 3. Detection of YHV by RT-PCR. Lane M: molecular weight marker. Sources of templates were Lane 1: total RNA extracted from 25 µl of hemolymph drawn from YHV-infected shrimp; Lane 2: 1 µl of hemolymph (without RNA extraction) from YHV-infected shrimp; Lane 3: 1 µl of hemolymph from uninfected shrimp. The oligonucleotides YHV273F/R were used as primers

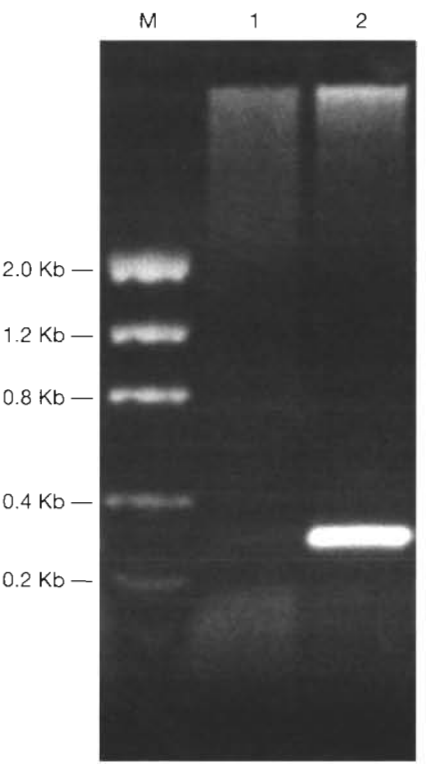

Fig. 4. Determination of viral strand sequence by RT-PCR. Lane: M: molecular weight marker; Lane 1: oligonucleotide YHV273F used as the primer for the RT reaction; Lane 2: oligonucleotide YHV273R used as the primer for the RT reaction. After the RT reaction, the complementary primer was added prior to PCR amplification as described in the 'Materials and methods'. Total RNA isolated from 25 µl of cell-free hemolymph drawn from YHV-infected shrimp was used as the template

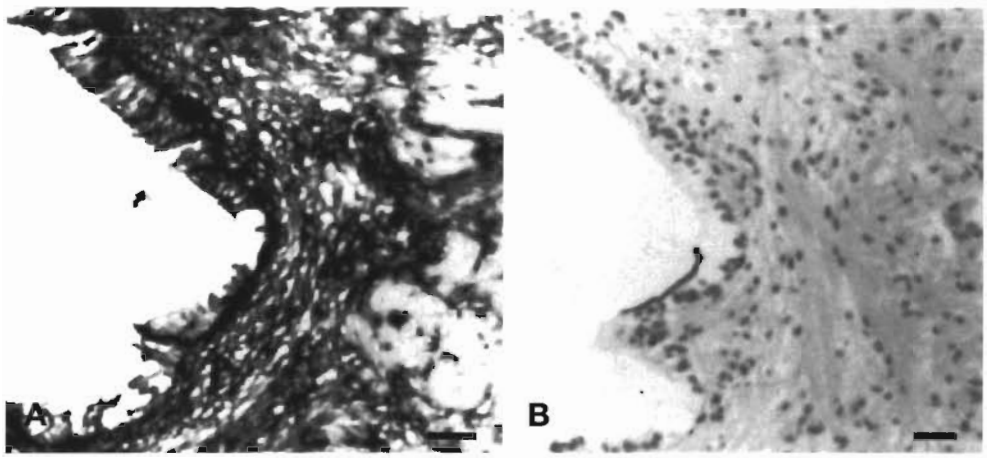

Fig. 5. *In situ* hybridization with YHV-infected shrimp tissues using single-stranded RNA (ssRNA) probes. (A) Tissue section hybridized with ssRNA synthesized using T7 RNA polymerase and *Sma* I-linearized template. (B) Tissue section hybridized with ssRNA synthesized using SP6 RNA polymerase and *Pst* I-linearized template. Both ssRNA probes were labeled with digoxigenin-11-UTP. The tissue shown is cuticular epithelium of the stomach. Scale bars = 40 µm

from *in situ* hybridization correspond to those of Lu et al. (1995) from viral titration in lymphoid organ culture.

For RT-PCR, the use of a small quantity (e.g. 1 µl) of shrimp hemolymph as the viral RNA template eliminated time-consuming RNA purification procedures and provided a rapid, sensitive detection method for YHV using primers for the 273 bp amplicon. In experiments with primers for larger amplicons based on the YHV cDNA clone (i.e. 1051 and 644 bp), less satisfactory results were obtained (data not shown). These larger PCR fragments were not obtained in non-nested RT-PCR reactions. The unsatisfactory results could have resulted from the YHV RNA being fragmented after RNA purification or from the rTth DNA polymerase being less efficient in transcribing larger sizes of YHV RNA. In a nested RT-PCR reaction, the 644 bp PCR amplicon could be obtained when using primers for the 1051 bp amplicon in the first RT-PCR followed by a second round of PCR using inner primers for the 644 bp amplicon. Larger PCR amplicons would facilitate study of sequence variations amongst various YHV isolates.

Our results from RT-PCR, ssRNA *in situ* hybridization and sequence analysis suggested that YHV is a positive sense RNA virus. The presence of large open reading frames in 2 additional clones (3-01 and 3-37) also supported this contention (data not shown). If this analysis is correct, then the placement of YHV in the family *Rhabdoviridae* is doubtful, since it comprises negative sense ssRNA viruses (Murphy et al. 1995). However, the polarity of the viral strand remains to be determined by synthesizing and identifying the polypeptides from the cloned DNA. A search in GenBank did not reveal any sequences homologous to that of the YHV cDNA described herein. This may be due to the fact that very few viral sequences of crustacean viruses have been submitted or that YHV is only distantly related to RNA viruses of other plant, invertebrate or vetebrate groups. We are continuing to screen and analyze clones that contain sequences adjacent to 3-27.

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